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The p53 tumor suppressor protein is a transcriptional activator and its transcriptional activity is required for p53 tumor suppression. We show here that p53 markedly activates expression of cyclin D1 and the induction of cyclin D1 is at least partially mediated by p21, a target of p53. To further understand features of p53 that contribute to cell cycle arrest and apoptosis several p53-null cell lines were generated that inducibly express wild-type or mutant forms of p53. Our results show that the cellular level of p53 can dictate the response of the cell to undergo either cell cycle arrest or apoptosis and DNA damage can heighten the apoptotic response to p53. We also demonstrate that a full apoptotic response to p53 requires both its N- and C-termini. In addition, we identify a novel activity within amino acids 43-63 which can activate transcription and mediate apoptosis. Furthermore, we show that the N-terminus of p53 is the target for destabilization mediated by ubiquitin and calpain and phosphorylation at serine 15 is in part attributable to stabilization following DNA damage.

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FOREWORD

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INTRODUCTION

Mutation of the p53 tumor suppressor gene occurs in about half of human cancers, including breast cancers [Hollstein et al. 1991; Ko and Prives 1996]. Furthermore, p53 may be functionally inactivated by other mechanisms, such as by the mdm2 protein which blocks its transcriptional activity [Oliner et al. 1993] or the E6 oncoprotein of human papillomavirus which facilitates degradation of p53 through the ubiquitination pathway [Scheffner et al. 1993]. Taken together, p53 inactivation is clearly a critical event in carcinogenesis.

p53 serves as a checkpoint in maintaining genome stability [Kastan et al. 1991; Lane 1992]. Loss of p53 in cells results in altered cell cycle regulation and a high degree of gene amplification, both of which contribute to abnormal proliferation and cellular transformation. Consistent with this idea, mice nullizygous for the p53 gene (p53-/-) are highly susceptible to tumor formation [Donehower et al. 1992], and aneuploidy and chromosomal instability are found in tumors from p53 -/- mice [Harvey et al. 1993]. Cells from patients with Li-Fraumeni syndrome (LFS) are heterozygous (mutant/wild-type) at the p53 gene locus and LFS patients are predisposed to multiple primary tumors [Young et al. 1981]. Loss of wild-type p53 in fibroblasts from patients with Li-Fraumeni syndrome results in altered cell cycle arrest and high gene amplification potential [Livingstone et al. 1992; Yin et al. 1992]. Furthermore, introduction of wild-type p53 into such cells can restore cell cycle control and inhibit gene amplification [Livingstone et al. 1992; Yin et al. 1992].

Following genotoxic or cytotoxic stresses such as the presence of damaged DNA, the cellular levels of p53 increase, resulting in at least three well understood responses: cell cycle arrest, differentiation, or apoptosis [Ko and prives 1996; Gottlieb and Oren 1996]. Several factors have been shown to determine how a cell responds to the accumulation of p53, including (1) cell type [Gottlieb and Oren 1996], (2) the transformation status of the cell [Lowe et al. 1993; 1994], (3) its microenvironment [Boudreau et al. 1995; 1996], (4) the presence of several cellular and viral proteins [Fisher 1994; White 1995]. Notably, the levels of p53 in a given cell can dictate the response of the cell such that lower levels of p53 result in cell cycle arrest [Chen et al. 1996; also see this report] or differentiation [Ronen et al. 1996] whereas higher levels result in apoptosis [Chen et al. 1996; Ronen et al. 1996; also see this report].

Cell cycle arrest. p53 is a sequence specific transcriptional activator of genes which contain its response elements [review by Vogelstein and Kinzler 1992]. A number of transcriptional targets of p53 have been identified. One of these is the cyclin-dependent kinase inhibitor p21 [El-Diery et al. 1993; Smith et al. 1994; Harper et al. 1993; Xiong et al. 1993]. Upregulation of p21 expression inhibits the protein kinase activities of G1 cyclin/CDK complexes and prevents phosphorylation of the retinoblastoma (RB) protein, thus preventing the cell from entering S phase [Slobos et al. 1994]. Overexpression of p21 can arrest cells in G1 [Chen et al. 1996; Harper et al. 1995; Brugarolas et al. 1995; Deng et al. 1995] or induce differentiation [Liu et al. 1996; Missero et al. 1996], depending on the cell type. Mouse embryo fibroblasts nullizygous for the p21 gene show a partial defect in G1 arrest that is less severe than that of p53-defective fibroblasts [Brugarolas et al. 1995; Deng et al. 1995], suggesting that p21 is only one of the genes that mediate p53-induced cell cycle arrest and that other p53-dependent G1 arrest pathway(s) may exist. Furthermore, many studies indicate that p53 can arrest cells in G2 [Ko and Prives 1996] and acts as a mitotic checkpoint protein [Cross et al. 1995]. Thus, additional p53 targets that mediate p53-dependent arrest in G1, G2 and/or the mitotic checkpoint remain to be identified.

Apoptosis. How does p53 induce apoptosis? Many studies, including our own, show that p53 transactivation activity contributes to the ability of p53 to induce apoptosis [Ko and Prives 1996; Chen et al. 1996; Sabbatini et al. 1995; Attardi et al. 1996; and also see this report]. There are at least two candidate genes which play roles in apoptosis that can be transactivated in response to p53 induction. In murine cells, p53 upregulates expression of the bax gene [Miyashita and Reed

1994], whose product dimerizes with bcl-2 and prevents the ability of bcl-2 to block apoptosis [Oltvai et al. 1993]. It is possible, therefore, that transcriptional activation of the bax gene by p53 induces apoptosis in murine cells. However, the bax gene is not activated by p53 in either human Saos-2 osteosarcoma cells or H1299 small cell lung carcinoma cells [Chen et al. 1996]. This may reflect the differences between species and/or cell types. A second p53 target that might influence apoptosis is insulin-like growth factor binding protein 3 (IGFBP-3) [Buckbinder et al. 1995]. IGFBP-3 is an antagonist of insulin-like growth factor-1 (IGF-1) and downregulation of IGF-1 or the IGF-1 receptor is correlated with the apoptotic response [Baserga 1994]. Because IGFBP-3 is a secreted protein its apoptosis inducing effects might be expected to affect neighboring cells that do not express p53 and/or IGFBP-3. However, only cells that express high levels of p53 undergo apoptosis and neighboring cells not expressing p53 do not undergo apoptosis. Thus, IGFBP-3 may have a function other than direct mediation of p53-dependent apoptosis. Therefore, additional p53 target genes responsible for p53-dependent apoptosis remain to be identified.

Several recent studies have provided evidence that p53 may have a transcription-independent function in apoptosis. p53-dependent apoptosis was shown to occur in cells treated with RNA and protein synthesis inhibitors [Caelles et al. 1994; Wagner et al. 1994]. Moreover, transactivation-defective point mutant and truncated p53 proteins can induce apoptosis in the human HeLa cell line [Haupt et al. 1995]. Other experimental results have led to the conclusion that transactivation by p53 is required for its role in apoptosis in rodent cells [Sabbitini et al. 1995]. Thus, different reports have provided essentially contradictory results as to the requirement for the sequence-specific transactivation function of p53 for induction of apoptosis. It is likely that these differences are dependent on the cell type employed in each studies. Consistent with the idea that p53 has a transcription-independent function in inducing apoptosis, osteosarcoma Saos-2 cell lines inducibly expressing transactivation deficient p53 (gln22-ser23) undergo apoptosis but not arrest upon p53 induction [Chen et al. 1996; also see this report]. Whereas cell lines expressing p53(Δ364-393), which lacks the C-terminal 30 amino acids, undergo cell cycle arrest and partial apoptosis following p53 induction, the cell line expressing a double mutant p53 (gln22-ser23Δ364-393), which has a mutated N-terminus (gln22-ser23) and a truncated C-terminus, fails to undergo apoptosis following p53 expression [Chen et al. 1996; also see this report]. Thus, the C-terminal 30 amino acids of p53 are not required for p53 transactivation activity, but required for p53 transcription-independent apoptosis. This notion is further supported by a recent study that the XP-B and XP-D DNA helicases, potential components of the p53-mediated apoptotic pathway [Wang et al, 1996], interact with the p53 C-terminus [Wang et al. 1995; Xiao et al. 1994; Leveillard et al. 1996]. Therefore, there is an alternative transcription-independent function of p53 in inducing apoptosis besides transcriptional activation and thus it is important to further define the domain(s) or region(s) of the protein responsible for this function.

p53 domains: structure and function. p53 has been subjected to extensive analysis of its functional domains [Gottlieb and Oren 1996; Ko and Prives 1996]. The p53 polypeptide consists of an activation domain, a sequence specific DNA binding domain located within the central, conserved portion of the protein, and, within the C-terminus, a nuclear localization signal, a tetramerization domain as well as a non-specific DNA binding domain (Figure 1).

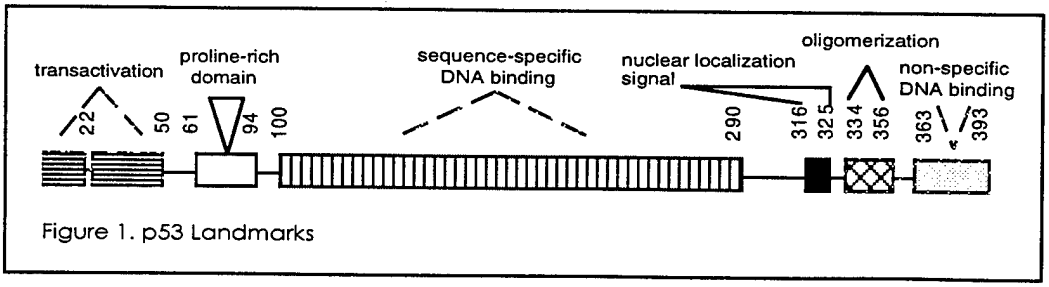

Figure 1. p53 Landmarks

The N-terminal 50 amino acids have been shown to be required for both transcriptional activation and repression [Lin et al. 1994; Ko and Prives 1996; Murphy et al.1996; Gottlieb and Oren 1996]. Mutation at residues 22 and 23 (gln22 and ser23) diminishes p53 function as both a transcriptional activator and a repressor [Lin et al. 1994; Murphy et al.1996]. This is presumably because these residues are required for the interaction of the activation domain with the TATA box binding protein associated factors (TAFs) [Lu and Levine 1995; Thut et al. 1995]. While the p53 transcriptional activity is absolutely required for p53-dependent cell cycle arrest and contributes to p53-dependent apoptosis, it appears that p53-dependent apoptosis also occurs independently of such p53 activity albeit somewhat delayed [Chen et al. 1996; Haupt et al. 1995]. However, it is not clear whether the activation domain has other function(s) which may be required for p53 transcription-independent apoptosis.

The central core region of p53 contains the sequence-specific DNA binding domain [Ko and Prives 1996; Gottlieb and Oren 1996; Vogelstein and Kinzler 1992]. The vast majority of the missense mutations that have been detected in tumors map to the central DNA binding domain of p53 [Hollstein et al. 1991; Ko and Prives 1996]. Among these are a number of mutational "hot spots" such as his175, trp248, ser249, his273 that occur with unusually high frequency and together make up about 40% of all tumor derived p53 mutations. Most mutations within the central DNA binding domain impair or abolish sequence-specific DNA binding by p53 [Vogelstein and Kinzler 1992]. In addition, mutations in this region lead to increased tumorigenic potential of cells harboring such mutant forms of p53 [Ko and Prives 1996; Gottlieb and Oren 1996; Vogelstein and Kinzler 1992]. Although the transactivation deficient p53 can induce apoptosis, the DNA binding domain is necessary for all known p53 functions, including apoptosis [Chen et al. 1996; Sabbatini et al. 1995; Attardi et al. 1996]. This suggests that in order to induce apoptosis, p53 either needs to bind to DNA or requires the DNA binding domain to maintain its competent apoptosis-inducing conformation.

The region in the extreme C-terminal 30 amino acids binds DNA non-specifically and acts as an autoinhibitory domain based on the observation that deletion of this region of p53 strongly stimulates DNA binding in vitro [Hupp et al. 1992]. Other manipulations of this region have similarly been found to stimulate DNA binding activity such as (1) phosphorylation of this domain by protein kinases casein kinase II [Hupp et al. 1992] or protein kinase C [Takenaka et al. 1995], or (2) by an interaction of this region with several different molecules such as p53 specific antibody [Hupp et al. 1992; Halazonetis et al. 1993], the p53 C-terminal peptide [Hupp et al. 1995], or the bacterial heat shock protein dnaK [Hupp et al. 1992]. Furthermore, the p53 transcriptional ability in vivo is enhanced by p53 specific antibody, or the p53 C-terminal peptide [Hupp et al. 1995; Abarzua et al. 1995]. Therefore, it is believed that this region controls ability of the protein to allosterically switch from "latent" to active for sequence-specific DNA binding and in turn, for transcriptional activation. Furthermore, this region of p53 also appears to have an additional function in mediating transcription-independent apoptosis since p53 lacking this domain was shown to have diminished ability to induce transcription-independent apoptosis [Chen et al. 1996; Wang et al. 1996]. The interaction of this region with the XPB and XPD DNA helicases may contribute to these effects [Wang et al. 1996].

p53 and chemotherapy. How do antineoplastic therapies work? The long-standing dogma is that such treatments are effective against cancers because they selectively kill rapidly dividing cells. Consistent with this model, human tumors that are most susceptible to such treatments are those with a high percentage of cells in the process of division, and normal tissues that proliferate rapidly (bone marrow, hair follicles and intestinal epithelium) are subject to damage by some of these potent antineoplastic agents. However, this explanation is not satisfactory since some curable cancers are slowly growing and some rapidly growing cancers are resistant to treatment [Fisher et al. 1994]. Recent advances in the understanding of apoptosis in antineoplastic

therapies suggests that such treatments kill certain cancers but not others due to the threshold of the apoptotic response of these tumor cells [Fisher et al. 1994]. The threshold is controlled by many factors, such as oncogene expression and cell type [Gottlieb and Oren 1996; Fisher 1994]. Importantly, the status of the p53 gene in tumor cells is the single most consistent prognostic indicator for cancer treatment outcome. Many studies have shown both in vitro and in vivo that cells with wild-type p53 are generally more sensitive to ionizing radiation and chemotherapy than cells that carry no p53 or mutant p53 [Lowe et al. 1993; 1994; Tsang et al. 1995; Lee and Bernstein 1993; Fan et al. 1994; Chresta et al. 1996]. The underlying mechanism for such outcomes may simply be due to the fact that p53 induces apoptosis in tumor cells [Ko and Prives 1996; Gottlieb and Oren 1996; Wu and Levine 1994].

How does p53 induce apoptosis in tumor cells following antineoplastic therapies? The simple explanation is that ionizing radiation and some genotoxic agents induce DNA damage in cells and through an unknown mechanism p53 senses the damaged DNA and is stabilized [Lu and Lane 1993; Kastan et al. 1992; Maltzman and Czyzyk 1984]. The resulting accumulation of p53 will either arrest cells or induce apoptosis, dependent upon the level of p53 accumulated, cell type and other factors [Ko and Prives 1996; Gottlieb and Oren 1996; Chen et al. 1996]. Furthermore, it is also possible that the efficacy of antineoplastic therapies depends upon the cooperation between p53-dependent and p53-independent apoptotic pathways activated by DNA damage [Peled et al. 1996]. In order to improve the efficacy of the cancer therapeutic drugs and to develop new strategies to treat cancers, it is important to understand the mechanism of p53 cooperation with antineoplastic drugs in inducing apoptosis.

p53 stability. In normal cells, the tumor suppressor protein p53 is maintained at low, barely detectable level with a half-life of about 30 minutes. In response to a variety of genotoxic agents and stress, the p53 protein is greatly stabilized, and the intracellular level increases dramatically. Although the phenomenon has been known for years, how genotoxic assaults signal to p53 remains elusive. At least two pathways have been shown to be involved in regulating the stability of p53 *in vivo*: one mediated by the calcium-dependent protease calpain (Pariat et al. 1997; Kubbutat et al. 1997); the other by the ubiquitination/proteasome degradation pathway (Maki et al. 1996; Chowdary et al. 1994). p53 was shown to be ubiquitinated in cells (Maki et al. 1996), and was found to be accumulated in a murine mutant cell line that is defective in the ubiquitin/proteasome pathway (Chowdary et al. 1994). In addition, p53 can also be induced by treating cells with drugs that inhibit either calpain (Pariat et al. 1997; Kubbutat et al. 1997) or the proteasome (Kubbutat et al. 1997; Maki et al. 1996).

One approach to dissect the DNA damage signaling pathway is to identify the domain(s) in p53 that contributes to its response toward the calpain/proteasome inhibitors, and is therefore likely to mediate the regulation of its stability and its inducibility upon DNA damage in cells. Additionally, since p53 is phosphorylated in cells, a possible involvement of DNA repair-related protein kinases in the signaling pathway was also examined.

BODY

The p53 tumor suppressor protein is a transcriptional activator and its transcriptional activity contributes to its ability to induce cell cycle arrest and apoptosis following various stress conditions. We have proposed to identify p53 targets that may play a role in p53 tumor suppression. By using immunoprecipitation, we have identified cyclin D1 as one of the p53 targets.

Accumulation of the wild-type p53 protein induces cyclin D1 expression.

Several studies have provided evidence that cyclin D1 is a key regulator of the G1 phase of the cell cycle (see review by Sherr 1993). We therefore considered the possibility that cyclin D1 may be regulated by p53. A p53-inducible system was used to analyze effects of the wild-type p53 protein on cyclin D1 expression. The glioblastoma cell line T98G, which was the parental cell line to establish the p53-inducible cell line GM47-23, contains an endogenous mutant p53 (met237 to ile) (Ullrich et al., 1992). GM47-23 cells contain the same endogenous mutant p53 gene and an exogenously introduced wild-type p53 gene under the control of a steroid responsive promoter (Mercer et al., 1990a). The related line, Del4A, contains the same endogenous mutant p53 and an additional dexamathasone-inducible deletion mutant p53 gene (Lin et al., 1992a). In the presence of dexamathasone, a moderate amount of inducible p53 was expressed (Fig. 2, compare lanes 4 and 6 with lanes 3 and 5, respectively) as previously reported (Mercer et al., 1990a). We confirmed that the inducible wild-type p53 protein adopts a unique conformational state *in vivo* which retains the p53-specific antibody PAb1801 epitope but very little or no PAb421 and PAb122 epitopes (Ullrich et al., 1992). This unique conformation and phosphorylation state presumably enables the inducible wild-type p53 protein to escape from the dominant negative effect of the endogenous mutant p53 (Milner and Mecalf 1991). To ascertain that the inducible wild-type p53 protein can transactivate its target gene in the GM47-23 cell line, expression of p21, a known p53-responsive gene (El-Deiry et al. 1993), was determined by immunoprecipitation. The p21 protein was detected only after dexamathasone treatment (Fig. 2, compare lanes 7 and 8). Note that there was a substantial amount of cyclin D1 detected in the immunoprecipitate after induction.

To examine whether p53 induction affects cyclin D1 GM47-23 cells were ^{35}S-methionine labeled at 0, 4, 8, 12, 24, and 48 h after treatment with dexamathasone and the amount of the cyclin D1 protein was determined by either immunoprecipitation using a specific anti-cyclin D1 antibody or coimmunoprecipitation using a specific antibody directed against p21 (Fig. 3A). The amount of cyclin D1 protein was markedly increased over the time course examined, peaking at 24h (compare lanes 1 and 7 with lanes 2-6 and 8-12, respectively). A similar kinetic pattern was observed for p21 expression (Fig. 3A, compare lane 1 with lanes 2-6). Induction of wild-type p53 in GM47-23 cells treated with dexamathasone was determined over a similar time course by immunoblot analysis (Figure 3B). Inducible wild-type p53 was detected between 8 to 12 h following treatment consistent with the kinetics of both cyclin D1 and p21 induction.

We also asked whether the induction of cyclin D1 expression by p53 is evolutionarily conserved by comparing human and rodent cell lines. A mouse cell line (3-4) was generated by transfection of mouse p53-null embryo fibroblasts (10-1) (Harvey and Levine 1991) with the mutant murine (ala 135 to val) p53 and was similarly examined. At 37^{0}C, the murine (val135) p53 is in mutant conformation and the cells grow, while at 32^{0}C, it is in wild-type conformation and the cells are arrested (Michalovitz et al. 1990; Martinez et al., 1991; and data not shown). Significantly more cyclin D1 protein was detected at 32^{0}C than that at 37^{0}C (Fig. 3C). No increase in cyclin D1 was observed at 32^{0}C in 10-1 cells (th parental cell line from which 3-4 cells were derived) (Fig. 3D). Thus, cyclin D1 induction by p53 occurs in both human and rodent cells.

To confirm the cyclin D1 response to wild-type p53 we also examined the effects of dexamethasone on T98G and Del4A cells (Figure 4 A,B and C). Since cyclin D1 is ubiquitously expressed at all stages of cell cycle a moderate amount of cyclin D1 was detected even in the absence of dexamathasone treatment of GM47-23 cells (Fig. 4A, lane 1). After dexamathasone treatment the amount of cyclin D1 was significantly increased as in the experiment shown in Fig. 2 (Fig. 4A, compare lanes 1 and 3 with lanes 2 and 4). Both in T98G cells which express only endogenous mutant p53 protein (Fig. 4B, lanes 1-2), and in Del4A cells which express endogenous mutant p53 and inducible deletion mutant p53 (Fig. 4C, lanes 1-2), p21 was not induced in the presence of dexamathasone treatment (Fig. 4B and 4C, compare lanes 3 and 4). Similarly, the amount of cyclin D1 protein was unchanged or slightly decreased in the presence of dexamathasone treatment compared with that in the absence of dexamathasone treatment (Fig. 4B and 4C, compare lanes 5 and 6).

We also examined the effect of p53 induction on other cyclins and cdks (Fig. 5). When the steady-state levels of other cell cycle related proteins were determined by immunoprecipitation, it was clear that the wild-type p53 protein either inhibited expression of CDK4 (Fig. 5A, compare lanes 1 and 2), CDK2 (Fig. 5A, compare lanes 3 and 4), cdc2 (Fig. 5A, compare lanes 5 and 6), or cyclin B1 (Fig. 5A, compare lanes 7 and 8), or had no significant effect on PCNA (Fig. 5A, compare lanes 9 and 10). Similarly, Western blot analysis showed that, relative to the amount of actin protein detected, the level of the cyclin A protein was slightly decreased in the presence of the wild-type p53 protein in the GM47-23 cell line (Fig. 5B, compare lane 1 with lanes 2-6). This is consistent with a recent report that p53 inhibits cyclin A expression (Yamamoto et al., 1994) although the promoter of the cyclin A gene contains a putative p53 responsive element (Yamamoto et al., 1994; Wang and Tjian 1994).

A DNA damaging agent causes increased levels of p53, p21 and cyclin D1.

To further characterize the role of cyclin D1 in p53 dependent cell growth control, we asked whether cyclin D1 is a part of the p53-dependent DNA damage response pathway. RKO cells contain wild-type p53 which arrest in G1 following irradiation (Kuerbitz et al. 1992; Leonardo et al. 1994). After treatment with 300 nM/ml of camptothecin, a topoisomerase inhibitor which induces DNA strand breaks in cells (Nelson and Kastan 1994), cells were ^{35}S-labeled and the amounts of the p53, p21 and cyclin D1 proteins were determined by immunoprecipitation. Fig. 6A showed that DNA damage resulted in a 5 to 10-fold increase of the wild type p53 protein (compare lanes 1 and 2). An increase of wild-type p53 in the DNA damaged cells was commensurate with induced p21 and cyclin D1 expression (Fig. 6B, compare lanes 1 and 3 with lanes 2 and 4, respectively). Those observations were consistent with the results obtained above (Fig. 2-4) from GM47-23 cells in that accumulation of wild-type p53 increases both p21 and cyclin D1 synthesis.

p53-dependent induction of cyclin D1 is at the transcriptional level.

To determine whether accumulation of the wild type p53 protein stabilizes the cyclin D1 protein, the half-lives of the cyclin D1 protein in the p53-inducible cell line GM47-23 and the normal human cell line WI-38 were determined. From the results obtained it was clear that the cyclin D1 protein has same half-life (approximately 15-30 min) both in the GM47-23 cell line either in the presence or in the absence of the wild-type p53 protein and in another human cell line (WI-38 cells) (data not shown). These data are consistent with previously published results (Matsushime et al. 1991). Thus, induction of cyclin D1 by p53 is not due to protein stabilization.

To analyze whether induction of cyclin D1 expression is at the transcriptional level, cyclin D1 mRNA was quantitated by Northern blot analysis (Figure 7). Poly(A)+ RNAs were isolated from the GM47-23 and T98G cell lines in the presence or absence of dexamathasone treatment. Using a labeled human cyclin D1 cDNA as probe, it was determined that the amount of cyclin D1

mRNA was actually slightly decreased in the first 8 h following dexamathasone treatment, but then increased between 8 and 12 h and peaked at 24 h (Fig. 7, top panel, compare lane 1 with lanes 2-6). PhosphorImage quantitation of several experiments showed that there was an over-all four-fold increase of cyclin D1 mRNA after dexamathasone treatment after normalization with GAPDH mRNA levels (Fig. 7, top and middle panel, compare lane 1 with lanes 2-6). This was consistent with the increase in protein levels observed. As a positive control, when the same blot was probed with human p21 cDNA p21 mRNA was shown to be gradually induced and peaked sharply at 24 h (Fig. 7, bottom panel, compare lane 1 with lanes 2-6). No induction of either cyclin D1 or p21 mRNA was observed in T98G cells (Fig. 7, all panels, compare lane 7 with lanes 8-11).

Since p53 induction of cyclin D1 synthesis is apparently transcriptionally regulated, we asked whether the cyclin D1 promoter contains a p53-responsive element. Our experiments showed that a 104 bp fragment [from -1235 to -1133 upstream of the first methionine (Herber et al, 1994)] contains a weak p53 binding site (data not shown). This binding appears to be specific as determined by competition gel-shift assays in which it was shown that a wild-type p53 DNA binding oligo (RGC) but not mutant oligo (mRGC) competed away p53 binding to the cyclin D1 promoter DNA fragment as well as by DNaseI protection. Examining the sequence of the p53-protected region by DNaseI footprinting analysis (data not shown) showed that there is a loose consensus p53 DNA binding site (El-Deiry et al., 1992; Funk et al., 1992), AAt tcAG TCC CA GGG CAAa TTC. However, when the 1.3kb cyclin D1 promoter DNA fragment was cloned upstream of a promoterless luciferase gene (CYCD1-LUC) and tested for transcriptional activity in dexamthasone treated and untreated GM47-23 cells we observed that this promoter reporter construct was induced only very slightly (2-4-fold) when compared to a positive control reporter with a 100-bp fragment containing the p53 DNA binding site from the GADD45 gene (Kastan et al, 1992) whose expression was stimulated by over 30-fold under the same conditions (data not shown). It remains possible that p53 can directly, albeit weakly, activate expression of the cyclin D1 promoter. However, an alternative explanation is that cyclin D1 induction by p53 may be mediated by one or more p53 responsive genes.

p21 expression induces cyclin D1

Since the p21 gene, itself a target of p53, is involved in cell cycle regulation, we asked whether induction of p21 can mediate the p53 dependent induction of cyclin D1 expression. To this end the human p21 cDNA was cloned downstream of a cytomegalovirus (CMV) immediate early gene promoter producing a p21 expression vector (pcDNA3-p21). Following transient transfection with pcDNA3-p21, T98G cells were ^{35}S labeled and the amounts of the p21 and cyclin D1 proteins were determined by immunoprecipitation (Fig. 8). As expected, with increasing amounts of transfected pcDNA3-p21 DNA, p21 protein was detected in a dose-dependent manner (compare lane 6 with 7), although the highest amount of transfected DNA reduced somewhat the transfection efficiency (compare lane 7 with lane 8). This increase in p21 was commensurate with significant increases in the amounts of the cyclin D1 protein in the transfected cells (compare lane 1 with lane 2-4). Furthermore, there were markedly greater quantities of cyclin D in the p21 immunoprecipitates (Fig. 8, compare lanes 2-4 with lanes 6-8, respectively). The fact that the cyclin D stimulation by p21 was relatively modest compared to what was observed with GM47-23 or 3-4 cells is most likely due to the fact that the efficiency of transient transfection is relatively low (around 5% of total cells), and therefore, it is probable that the induction of cyclin D1 was significantly greater than what we detected. We conclude that p21 mediates the induction of cyclin D1 expression by p53.

Induction of cyclin D1 is concomitant with a shift between the slow and the fast migrating forms of cyclin D1.

It has been reported that the cyclin D1 protein exists in two forms *in vivo*, comprising slow and fast electrophoretically migrating species, both of which are phosphorylated (Matsushime et

al. 1991; and 1994). To further elucidate the p53 effect on cyclin D1, we determined the steady state equilibrium between the slow and fast migrating forms of the cyclin D1 protein. In our experimental conditions, the two forms of the cyclin D1 protein in GM47-23 cells can be detected by Western blot analysis but not by immunoprecipitation. We used a rabbit polyclonal antibody (UBI) which mainly recognizes the fast migrating form of cyclin D1 in GM47-23 cells but both forms of cyclin D1 in 3-4 cells, and a monoclonal antibody (HD11) which recognizes both forms (data not shown). As the wild-type p53 protein was detected between 8 to 12 h in GM47-23 cells after dexamathasone treatment (Figure 3B), the equilibrium of the two cyclin D1 forms began to shift toward the fast migrating form (Fig. 9A. top and middle panels, compare lanes 3 and 4). Thus, the induction of cyclin D1 expression observed above (Figures 2-4) is primarily due to the induction of the fast migrating form (Fig. 9A, top and middle panels, lanes 5-6). Note that as more of the p53-induced fast migrating form of cyclin D1 was accumulated less of it was recognized by the monoclonal antibody (HD11), strongly suggesting that this p53-induced form underwent a conformational or post-translational modification (Fig. 9A, compare lanes 5-6 of the top panel with lanes 5-6 of the middle panel, respectively). A similar shift to the fast migrating form of cyclin D1 was seen in the mouse 3-4 cell line (Fig. 9B).

To further understand how p53 might regulate arrest vs. apoptosis we have used the p53 null cell lines Saos-2 and H1299, to generate lines inducibly expressing either wild-type or mutant forms of p53. These cell lines have allowed us to (a) identify a quantitative response to different amounts of p53, (b) examine a number of p53 variants in clonally derived cell populations in order to derive information about the influence of functional domains of p53 on cell arrest and apoptosis, and (c) determine the effect of DNA damage on p53 in this context. Our results have both provided new insight into p53 and should provide a paradigm for this approach to studying p53 structure and function.

The level of p53 within Saos-2 cells determines cell death or arrest.

To establish stable cell lines that inducibly express p53, the tetracycline-regulated expression system is used. In the presence of tetracycline in media, the p53 protein is not expressed. Upon withdrawal of tetracycline from media, p53 expression is induced within 8 h in cells. Of the two wild-type p53 inducible cell lines obtained, one of these (p53-7) expressed p53 protein at relatively high levels upon withdrawal of tetracycline and will be refered to as the "high p53 producer" line. Although high relative to other transfected clones, the amount of detectable induced p53 protein in p53-7 cells, however, was substantially less than that induced in DNA damaged human RKO cells that contain wild-type p53 (Kastan et al. 1992), demonstrating that the level of p53 expressed in the Saos-2 cell line is within the physiological range (data not shown). When the growth curve of induced and uninduced p53-7 cells was examined, there was a dramatic difference in cell viability between the two states, induced and uninduced. While the uninduced cells continued to grow with a doubling time of ~48 hrs, the p53 expressing cells started to die within 2 days (as determined by reduced cell count) and by 3 days, only 10% of the cells survived. By 5 days after induction there were virtually no detectable viable cells remaining on the plate (Fig. 10A).

The apoptotic cells showed many characteristics of the apoptotic process, such as chromosomal condensation and apoptotic bodies. Another characteristic of apoptotic cells is fragmentation of DNA (for review, see Wyllie 1985), and when such cells are fixed in ethanol, DNA fragments leak out of their nuclei. Fluorescence-activated cell sorter (FACS) analysis of the DNA content of apoptotic cells indicates that they have less than the normal content of G1 DNA (referred to as sub-G1). FACS analysis of induced p53-7 cells showed that 1 day after tetracycline withdrawal cells in S phase were reduced from 14.03% to 4.94%, and cells in G2 were increased from 14.37% to 22.68% (Fig. 10B), suggesting that a significant G2 arrest had occurred in at least

a fraction of the cells. At this time point little or no sub-G1 content cells were scored. By 2 days, however, 24.92% of the cells had a sub-G1 DNA content and at three days after induction more than 60.43% of the cells had sub-G1 DNA content, with the remaining cells primarily arrested in both G1 and G2 (Fig. 10B).

The other p53 inducible cell line (p53-13), which will be refered to as the "low p53 producer", expressed p53 protein at relatively low levels upon withdrawal of tetracycline. The amount of p53 expressed in p53-13 cells is approximately 25-50% of that detected in p53-7. In contrast to p53-7 cells, p53-13 cells showed substantially slowed cell growth, yet no reduction in cell number or appearance of cells with sub-G1 DNA content. FACS analysis showed that p53-13 cells exhibit a significant arrest in both G1 and G2, but no apoptosis (data not shown).

Since the only obvious difference between the p53-7 and p53-13 cells was the amount of p53 detected after induction, we tested whether the level of p53 was, in fact, capable of regulating the apoptotic vs. arrest responses. This was done by varying the amounts of tetracycline in the culture medium of the high producer p53-7 cells. The results showed that median to high concentrations of tetracycline allowed for a low expression of p53 while lesser amounts of tetracycline lead to greater accumulation of p53 (Fig. 10C). Cells were counted at day 0, 1, or 3 following induction of p53 to different extents. The number of surviving cells was generally both inversely proportional to the amount of p53 expressed and directly proportional to the amount of tetracycline present in the culture media (Fig.10D). There was evidence of some increase in cell number with the conditions resulting in the two lowest amounts of p53 induced (i.e. 40 and 20 ng/ml of tetracycline), however, it was significantly less than with no induction. Thus, intermediate levels of p53 caused slowed cell growth and cell arrest but not a significant amount of cell death, while high levels of p53 caused apoptosis. We conclude from these data that the level of p53 in the p53-7 Saos 2 cell line can determine whether the cells undergo growth arrest or apoptosis.

A full apoptotic response to p53 in tumor cells requires both sequence-specific transactivation and C-terminal regulatory domains of p53.

A p53 double mutant (gln22-ser23) was shown to be defective in transactivation [Lin et al. 1994], presumably due to the inability of this mutant to bind to TATA-box binding protein associated factors (TAFs) that are critical for p53 mediated activation [Lu and Levine 1995; Thut et al. 1995]. This mutant, however, has produced contradictory results as to whether transcriptional activation is necessary for p53-mediated apoptosis [Sabbatini et al. 1995; Haupt et al. 1995]. To determine whether apoptosis can occur through a p53 transcription-independent pathway in Saos-2 cells, a cell line, 22/23-4, which expresses high levels of inducible transactivation deficient p53 (gln22-ser23) was used. Consistent with evidence from in vitro studies [Lin et al. 1994] and transient transfection assays [Lin et al. 1994; 1995], this mutant form of p53 was transcriptionally inert since endogenous p21 was not induced even by high levels of p53 (gln22-ser23) [data not shown]. Consistent with the lack of induction of p21 in 22/23-4 cells, no detectable cell cycle arrest was observed after induction of mutant p53 as determined by FACS analysis (data not shown). Nevertheless, in these cells p53 (gln22-ser23) induced cell death, albeit, to a lesser extent and with delayed kinetics when compared to wild-type p53 (Fig. 11). Those cells which did not undergo apoptosis contained a normal S-phase DNA content and presumably kept cycling (data not shown). The apoptosis induced by p53 (gln22-ser23), although reduced, was significantly greater than either the background levels of cell death that occur in the presence of tetracycline, or in cell expressing mutants completely defective in apoptosis (Figure 11). Our data confirm and extend observations by Oren and colleagues [Haupt et al. 1995] who showed that apoptosis can be brought about by p53 mutants such as p53 (gln22-ser23) that are defective in sequence specific transactivation in transiently transfected HeLa cells. These data also provide clear evidence that the abilities of p53 to induce cell cycle arrest and apoptosis are genetically separable from each other.

p53 contains an autoinhibitory region within the last 30 amino acids of the protein. Deletion of this region generates a p53 protein that has higher DNA binding in vitro (Hupp et al. 1992) and that is comparable to full length p53 in activating transcription in transient transfection assays in cells (Halazonetis and Kandil 1993). To determine the cellular response to a p53 lacking the C-terminal 30 amino acids (p53Δ364-393), cell lines expressing this p53 variant were isolated. One of these lines, p53ΔC30-6, contained at least two-fold more p53 than the high producer cell line p53-7 when normalized to the cellular actin protein levels. Consistent with observations that p53(Δ364-393) mutant is functional in transactivation, p21 was induced in p53ΔC30-6 cells to a similar extent as that by wild-type p53. Unexpectedly, however, although the growth of p53ΔC30-6 cells was completely arrested upon induction, the cell number did not decrease detectably throughout the time course of the experiment, suggesting that only a small fraction of p53ΔC30-6 cells underwent apoptosis. Consistent with this idea, FACS analysis of ΔC30-6 cells taken over 4 days showed that upon induction of p53(Δ364-393), a weak apoptotic response with delayed kinetics ensued (Fig. 11). Therefore, in this cell line p53(Δ364-393) can induce apoptosis, but far more weakly than full-length p53, suggesting that the C-terminal 30 amino acids of p53 are required for efficient apoptotic activity. We first considered the possibility that the ΔC30-6 cells containing inducible p53(Δ364-393) had become intrinsically defective for p53 mediated apoptosis. However two points argue strongly against this. First, as seen with the low producer wild-type p53 cell line, p53-13, treating p53ΔC30-6 cells with camptothecin led to a marked increase in cell death (data not shown). Second, additional Saos2 and H1299 cell lines expressing p53(Δ364-393), were all similarly defective in apoptosis (see below). We therefore conclude that in Saos2 and H1299 cells a strong apoptotic response requires the intact C-terminus of p53.

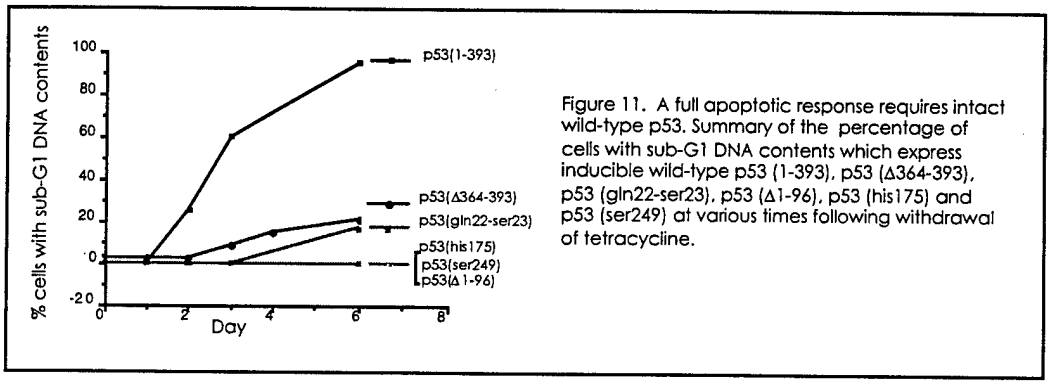

Figure 11. A full apoptotic response requires intact wild-type p53. Summary of the percentage of cells with sub-G1 DNA contents which express inducible wild-type p53 (1-393), p53 (Δ364-393), p53 (gln22-ser23), p53 (Δ1-96), p53 (his175) and p53 (ser249) at various times following withdrawal of tetracycline.

To gain further information as to whether the C-terminal domain of p53 would itself be sufficient to induce apoptosis in the absence of the N-terminus, a Saos-2 cell line ΔN96-5 expressing p53(Δ1-96) was used. ΔN96-5, which lacks the entire transactivation domain, is capable of sequence specific DNA binding (our unpublished observations) but is completely defective in sequence-specific transactivation (Pietenpol et al. 1994). Upon withdrawal of tetracycline, ΔN96-5 cells expressed markedly higher levels of truncated p53 than the high producer wild-type p53 cell line, p53-7 (data not shown), yet this induction had no measurable effect on cell growth or arrest (Fig. 11 and also see table 1). This indicates that neither DNA binding nor the C-terminal domain are sufficient for apoptosis.

Our results show that p53 with mutations within the N- and C- termini were inefficient but not completely inert in inducing apoptosis in Saos-2 cells. Tumor-derived mutant forms of p53 contain intact N- and C-termini, but are incapable of transactivating and binding specifically to p53 responsive elements (for reviews, see Gottlieb and Oren 1996; Ko and Prives 1996). In order to determine if such mutants would display any apoptotic activity in Saos-2 cells, cell lines which contained inducible mutant forms of p53 (ser249 or his175) were generated. Upon induction of high levels of either p53 (ser249) or p53 (his175), essentially no changes in growth or survival were detected in the Saos-2 cells as compared to either the uninduced state or the parental cell from

which they were derived (Fig. 11 and also see table 1). These results confirm that tumor derived mutant forms of p53 are inert for inducing the apoptotic response.

DNA damage can sensitize cells to p53-mediated apoptosis without affecting the level of p53 protein.

Effectors of DNA damage have been shown to increase the amount of p53 in cells by a post-transcriptional mechanism (Maltzman and Czyzyk 1984; Kastan et al. 1992; Lu and Lane 1993). Since the quantity of p53 induced was clearly a determinant of the switch between arrest and apoptosis in p53-7 cells, we wished to test whether the levels of p53 in the low producer cell line, p53-13, could be augmented after DNA damage, and whether the cells would now undergo apoptosis. Camptothecin (CPT), a topoisomerase inhibitor and cancer therapy drug, has been shown to induce DNA damage in cells (Nelson and Kastan, 1994). When p53-13 cells were treated with increasing amounts of CPT in the presence or absence of tetracycline we observed that even without p53 induction there was a modest apoptotic response to CPT suggesting that Saos-2 cells can undergo DNA damage-associated apoptosis in a p53- independent manner (Fig. 12). Unexpectedly, however, when p53 was induced in CPT-treated p53-13 cells there was both a significant increase in the number of apoptotic cells (Fig. 12), and yet no discernable increase of p53 protein levels (data not shown). This may be due to the difference of cell types because p53 is stabilized in LNCap cells following CPT treatment (see below). Thus p53 and CPT cooperate in Saos2 cells to cause a strong apoptotic response, and this occurs in a manner that is independent of p53 protein accumulation.

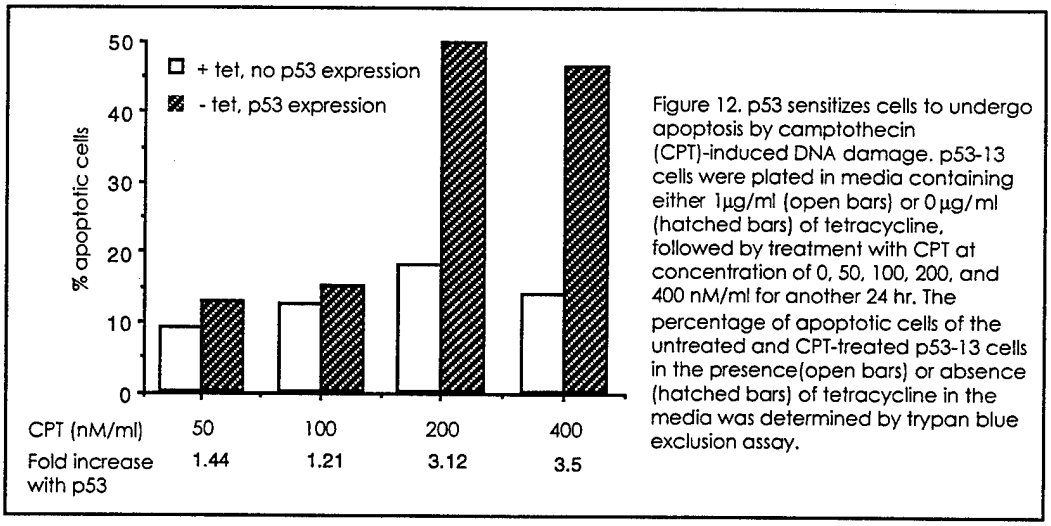

Figure 12. p53 sensitizes cells to undergo apoptosis by camptothecin (CPT)-induced DNA damage. p53-13 cells were plated in media containing either 1μg/ml (open bars) or 0μg/ml (hatched bars) of tetracycline, followed by treatment with CPT at concentration of 0, 50, 100, 200, and 400 nM/ml for another 24 hr. The percentage of apoptotic cells of the untreated and CPT-treated p53-13 cells in the presence(open bars) or absence (hatched bars) of tetracycline in the media was determined by trypan blue exclusion assay.

H1299 cells with inducible wild-type and mutant forms of p53 confirm and extend results in Saos2 cells.

During the course of the experiments described above with Saos2 cells it was decided to use the same strategy to generate a number of additional inducible cell lines in another p53-null human cell background. Initially, we chose both H1299 and the breast cancer cell line MDA-MB-453, both of which are p53-null. After several experiments, we failed to generate any stable inducible cell lines using MDA-MB-453. Instead, by using H1299 cells, a number of stable inducible cell lines were generated and the results are summarized in Table 1.

Table 1. Cell lines expressing inducible p53 or p21.

Protein expressed [a]	Saos-2 [b]	H1299 [b]	Apoptosis [c]	Arrest [c]	Cycling [c]
Wild-type p53 (H)	1	2	+++	+ [d]	-
(L)	1	5	-	+	-
p53 (gln22-ser23) (H)	3	2	+	-	-
(L)	3	3	-	-	+
p53 (Δ364-393) (H)	2	2	+	+	-
(L)	3	2	-	+	-
p53(gln22-ser23Δ364-393) (H)	nd	1	-	-	+
p53 (Δ1-96) (H)	4	1	-	-	+
p53 (ser249) (H)	4	4	-	-	+
p53 (his175) (H)	4	3	-	-	+
p21 (H)	3	3	-	+	-

(a) Proteins were detected by Western blotting with p53 specific monoclonal antibodies PAb1801 or PAb421, or p21 specific monoclonal antibody AB-1 (Oncogene Science). Clones were divided where possible into high (H) and low (L) producers when protein levels differed by at least two-fold.
(b) Number of individual clones of Saos-2 or H1299 cells expressing inducible p53 or p21.
(c) Apoptosis , arrest or cycling states of cells were determined by growth curves and FACS analysis.
(d) In cells which underwent massive apoptosis, cell arrest was transient.

As was observed with Saos2 cells, the high p53 producer H1299 cell lines underwent apoptosis after induction while the low producer H1299 cell lines underwent arrest. Consistent with results observed in Saos2 cells, H1299 lines expressing ser249, his275 or Δ1-96 mutant forms of p53 were completely unable to induce apoptosis (Table 1). Moreover, cells expressing the C terminally truncated mutant p53(Δ364-393) or the transactivation defective mutant p53(gln22-ser23), underwent apoptosis with reduced kinetics and extent over the time course examined. Importantly, a p53 variant that contained both the N-terminal double mutation at residues 22 and 23 but which also lacked the C-terminal 30 amino acids p53(gln22-ser23Δ364-393), although also expressed at high levels in H1299 cells, was completely inert in inducing apoptosis or growth arrest. This finding provided the strongest evidence that both N- and C-termini of p53 are required for apoptosis in tumor cells.

To further delineate the functional domains of p53 in the N-terminus, several new mutations were generated and stable cell lines that inducibly expressing such mutants were established. Analysis of these cell lines provides new insights about p53 functional domains in the N-terminus.

Deletion of the N-terminal 22 amino acids enhances the ability of p53 to induce apoptosis while deletion of the N-terminal 23 amino acids inhibits.

Previously, we showed that expression of p53(Δ1-22), which lacks the N-terminal 22 amino acids, as well as wild-type p53 and transactivation deficient p53(gln22-ser23) in p53 null cells can induce apoptosis (Chen et al. 1996). To further characterize the mutant p53(Δ1-22), we analyzed one of the high p53 producer cell lines, p53(Δ1-22)-3 (Fig.13A). First, the

transcriptional activation property of p53(Δ1-22) was determined by monitoring the expression level of the endogenous p21 gene, a known transcriptional target of p53. p53(Δ1-22) efficiently activated endogenous p21 expression (Fig. 13A). Next, the growth rate of the p53(Δ1-22)-3 cell line was determined (Fig. 13B). A majority of cells died within 3 days after induction of p53(Δ1-22) (Fig. 13B). It has been established that the percentage of cells containing sub-G_1 DNA content can measure the extent of cells undergoing apoptosis (Chen et al. 1996; Haupt et al. 1995; Sabbatini et al. 1995). Since p53 can induce apoptosis in H1299 cells in a variety of experimental protocols (Chen et al. 1996; Haupt et al. 1995), FACS analysis was done to determine the extent of apoptosis in cells expressing different p53 constructs. The results showed that 69% of cells expressing p53(Δ1-22) had sub-G_1 DNA content 3 days after plating, compared to only 6% of the control cells (Fig. 13C). Consistent with FACS analysis, approximately 65% of cells were dead as determined by trypan blue exclusion assay, indicating that either method gives a consistent, reliable index for the degree of apoptosis. In contrast, about 45% and 30% of cells had sub-G1 DNA content at 3 days following expression of wild-type p53 and transactivation deficient p53(gln22-ser23), respectively (data not shown; also see table 2).

Table 2. Characteristics of Various mutant p53 proteins

	SST [a]	Arrest [b]	Apoptosis [c]
wild-type p53	+++	+++	+++
p53(gln22-ser23)	-	-	++
p53(Δ1–22)	+++	+++	++++
p53(Δ1–23)	+	++	+
p53(Δ1–42)	++	++	++++
p53(Δ1–63)	-	-	-

(a) SST, sequence-specific transactivation, was assayed by the levels of p21 expression activated by p53.
(b) Arrest was assayed by the amount of the S-phase cells reduced by p53.
(c) Apoptosis was assayed by the amount of the sub-G_1 phase cells or trypan blue-stained cells caused by p53.

Extensive N-terminal amino acid substitution analysis by Lin et al (1994) indicated that residues 22 and 23 are critical for p53 transcriptional activity. Thus, it is interesting to determine whether p53(Δ1-23), which deletes the N-terminal 23 amino acids, can still be capable of inducing cell cycle arrest and/or apoptosis. Among nine stable cell lines established, at least three of them are high p53 producers as compared to a high wild-type p53 producer cell line, p53-3 (Fig. 14A). Interestingly, p53(Δ1-23) is still capable of activating expression of the endogenous p21 gene albeit at lower efficiency than wild-type p53 (Fig. 14A). Growth rate (Fig. 14B) and FACS (Fig. 14C) analyses of a high producer cell line, p53(Δ1-23)-9, showed that p53(Δ1-23) induces apoptosis at much lower capacity than both wild-type p53 and p53(Δ1-22) (see table 2). In addition, consistent with the result that p53(Δ1-23) can activate p21 expression, cells which did not undergo apoptosis primarily arrested in G_1 (Fig. 14C). Similar results were obtained using the cell line p53(Δ1-23)-10, another high p53(Δ1-23) producer.

The region within residues 43-63 contains novel activities which support transcription and promote apoptosis.

The previously defined p53 transcriptional activation domain was found to lie within residues 1-42. This was determined by analyzing transcriptional activation of GAL4 responsive promoter by GAL4 DNA binding domain-p53 activation domain fusion proteins (Fields and Jang 1990; Raycroft et al. 1990), and by analyzing activation of a reporter gene under control of a promoter with a p53 responsive element when p53 was cotransfected (Chang et al. 1995; Lin et al. 1994). Since p53(Δ1-23) is still capable of activating expression of the endogenous p21 gene and inducing cell cycle arrest and apoptosis, it is important to determine whether the other half (residues 24 to 42) of the previously defined activation domain is required for such activities.

To this end, 16 individual stable cell lines that inducibly express p53(Δ1-42), which lacks the N-terminal 42 amino acids and the entire previously defined activation domain, were established. Fig. 15A shows three representative cell lines. Surprisingly, p53(Δ1-42) is expressed at no higher than intermediate levels in 16 of these clonal cell lines when compared to wild-type p53 in the p53-3 cell line (Fig. 15A; and data not shown). Nevertheless, expression of the endogenous p21 gene in these cell lines was activated by p53(Δ1-42) , especially in the cell lines p53(Δ1-42)-2 and p53(Δ1-42)-5 (Fig. 15A). We then determined the growth rate of p53(Δ1-42)-2 cells and the ability of p53(Δ1-42) to induce cell cycle arrest and apoptosis. Growth rate analysis showed that a majority of cells died within 3 days (Fig. 15B). Consistent with this observation, both trypan blue exclusion assay and FACS analysis showed that approximately 68% of cells underwent apoptosis and surviving cells arrested in G_1 (Fig. 15C). Similar results were obtained from several other cell lines (data not shown). This strong apoptotic activity of p53(Δ1-42) may explain why only cell lines expressing intermediate levels of p53(Δ1-42) can be cloned since a basal level expression of the p53(Δ1-42) protein in high producer cells under the uninduced condition might have killed the cells and prevent them from multiplying. These results suggest that p53(Δ1-42) contains novel function which supports activation of the endogenous p21 gene and promote apoptosis.

To further delineate this novel p53 functional domain, we generated seven inducible cell lines expressing p53(Δ1-63), which lacks the N-terminal 63 amino acids but contains the intact proline-rich region. Fig. 16A shows four representative cell lines. When expression levels of the endogenous p21 gene in these cell lines were determined, the results showed that p53(Δ1-63) is unable to activate p21 expression (Fig. 16A). Consistent with this observation, the cell line p53(Δ1-63)-14, which expresses a high level of p53(Δ1-63), continues to multiply under the induced condition (Fig. 16B). Furthermore, FACS analysis showed that neither apoptosis nor cell cycle arrest were observed in cells expressing p53(Δ1-63) under induced condition (Fig. 16C).

In the above, we show that the cellular level of p53 can dictate the response of the cell to undergo either cell cycle arrest and apoptosis. Therefore, it is important to determine how p53 stability is regulated.

N-terminal 96 amino acids but not the C-terminal 30 amino acids are required for ubiquitin/calpain-mediated destabilization of p53.

Although an *in vitro* ubiquitination and degradation system, as proposed in SOW, provides simpler and more regulatable assays, we were not able to obtain reproducible results using the system. An alternative approach was taken, in which full-length wild type or different parts of p53 were HA-tagged, and introduced into cells by transfection. Cells were then either treated with the calpain/proteasome inhibitor N-acetyl-leu-leu-norleucinal (LLnL) or left untreated for two hours. Cell extracts were prepared and the expression of exogenously introduced, HA-tagged p53 proteins were detected by immunoprecipitation followed by Western using the antibody 12CA5 that recognizes the HA tag. Figure 17 shows the results of such experiment using a human prostate carcinoma cell line LNCaP that contains wild type inducible p53 (Nelson and Kastan 1994). In these cells, the exogenous HA-tagged full-length p53 was induced by the inhibitor LLnL, although to a lesser degree compared to the endogenous wild type p53 (Fig. 17A). Deletion of the C-terminal 30 amino acids had no effect on the induction, whereas deletion of the N-terminal 96 amino acids completely abolished the induction by LLnL(Fig. 17B). Furthermore, a domain containing only the N-terminal 96 amino acids can be induced by the inhibitor LLnL (Fig. 17B). These results suggest that the instability of p53 in cells is probably regulated by the N-terminal domain, and the C-terminal 30 amino acids are dispensable for such regulation. Since the inducibility of the N-terminal domain alone appeared to be weaker than the p53(Δ364-393) construct, additional contribution from the central domain cannot be excluded.

DNA-damage induced stabilization of p53 is in part attributable to phosphorylation at serine 15.

Many protein kinases have been shown to phosphorylate p53. To investigate the possible involvement of phosphorylation in the DNA damage signaling pathway, we initiated a collaboration with Dr. Yoichi Taya (National Cancer Research institute, Tokyo) to generate a series of antibodies that recognize different phospho-serine residues in the N-terminal domain of p53. One of the antibodies, anti-phospho-Ser15, recognized p53 isolated from cells treated with DNA damage agents such as γ-, UV irradiation, camptothecin (CPT, topoisomerase I inhibitor), or actinomycin D (Fig. 18A) but not p53 stabilized by the calpain/proteasome inhibitor LLnL (Fig. 18B), indicating that phosphorylation of serine 15 is induced by DNA damage. In addition, the kinetic of induction also coincided with stabilization of p53 (Fig. 18C).

Serine 15 has been shown to be a site of phosphorylation in p53 by the DNA-dependent protein kinase (DNA-PK) (Lees-Miller et al. 1992). The C-terminus of the catalytic subunit of DNA-PK contains a PI-3 kinase homology domain and its activity can be inhibited by the PI-3 kinase-specific inhibitor wortmannin (Hartley et al. 1995). To investigate whether serine 15 is directly involved in stabilization of p53 following DNA damage, cells were treated with CPT in the presence or absence of wortmannin. As shown in Fig 18D, stabilization of p53 as well as phosphorylation of serine 15 was effectively, although not completely, inhibited by wortmannin. We are now testing the DNA-PK-defective murine cell line scid and a DNA-PK-null human glioblastoma cell line M059J to see if DNA-PK is involved in the phosphorylation of serine 15 in cells in response to DNA damage. Alternatively, serine 15 may also be phosphorylated by other yet to be identified PI-3 kinase family member.

Charges introduced by phosphorylation is not sufficient in stabilizing p53.

To investigate whether increased charges as a result of phosphorylation is the sole factor in stabilizing p53, serine 15 was mutated to aspartate in the context of HA-tagged p53(Δ364-393). The HA-tagged D15 mutant was then used in transfection assays to test its inducibility by LLnL. The results clearly indicate that the D15 mutant is as inducible by LLnL as the wild type p53 (Fig. 19), suggesting that increased negative charges alone are not sufficient in stabilizing p53. Instead,

a more complex issue involving the actual presence of the phosphate group is important in regulating the stability of p53.

Although our results implicate that phosphorylation at serine 15 contributes to the stabilization of p53, they by no means exclude the possibility that other N-terminal kinase(s) may cooperate with the serine 15 kinase in stabilizing p53. In fact, partial inhibition observed in wortmannin experiments (Fig. 18D) suggests the possibility that other events may also be involved.

CONCLUSION

Cyclin D1 is a component of G1-checkpoint and a mediator of p53 tumor suppression.

We have provided evidence that accumulation of the wild-type p53 protein leads to induction of cyclin D1 expression. This induction is mediated at least in part by the p21 gene product. Examination of the cyclin D1 protein that was induced by p53 revealed that it differs from the cyclin D1 found in actively cycling cells in several aspects. Stimulation of cyclin D1 expression by p53 is manifested by the preferential increase in a rapidly migrating form of the cyclin polypeptide. It is not yet clear what the precise physical differences are between the slow and fast migrating forms. It is likely that two forms of cyclin D differ in their state of phosphorylation. Although Matsushime et al. (1991) showed that both forms of cyclin D1 are phosphorylated they may differ in the extent to which they are thus modified. Indeed, it is attractive to consider that the more rapidly migrating form is underphosphorylated a condition resulting from the increased levels of the cdk inhibitor, p21, that is induced by p53.

As a G1 cyclin, cyclin D1 was originally identified by very different approaches: as a suppressor of yeast G1 cyclin mutations (Xiong et al., 1991; Lew et al., 1991), as a delayed early response gene induced by colony-stimulating factor 1 (Matsushime et al., 1991) and as a putative pro-oncogene BCL1/PRAD1 (Motokura et al., 1991; Withers et al., 1991; Rosenberg et al., 1991; Seto et al., 1992; Schuuring et al., 1992; Williams et al., 1992). It has been reported that overexpression of cyclin D1 promotes cell progression and differentiation, generally observed as shortened G1-S transition (Baldin et al., 1993; Quelle et al., 1993; Resnitzky et al., 1994; Musgrove et al., 1994) and oncogenesis (Bodrug et al., 1994; Wang et al., 1994). In addition, both activated ras and myc oncogenes induce cyclin D1 expression (Filmus et al. 1994; Daksis et al. 1994), suggesting that cyclin D1 is one of the mediators of oncogenic transformation. However, in some cases, increased levels of cyclin D1 have been a characteristic of arrested cells. Pagano et al. (1994) showed that transient overexpression of cyclin D1 in fibroblasts arrests cells in G1 phase. This arrest can be abolished by coexpression of cyclin D1 with PCNA, but not with CDK4 or CDK2. Overexpression of cyclin D1 inhibited PCNA relocation as well as PCNA-dependent DNA repair and replication, suggesting that cyclin D1 is a component of G1-checkpoint. Furthermore, Dulic et al (1993) and Lucibello et al (1993) have found that levels of cyclin D1 are increased in senescent cells, corresponding to the presence of non-functional cdks. Thus, it appears that increased levels of cyclin D1 can be found in two opposing aspects of cell cycle control: growth promoting and growth arresting. In a p53-inducible system, we showed that accumulation of wild-type p53 induces cyclin D1 synthesis and pRB becomes underphosphorylated. We can suggest that the effect of cyclin D in cells may reflect the status and levels of p53. Indeed, cyclin D1 may be a mediator of p53 growth suppression, consistent with one of the cyclin D1 functions in cell cycle control.

Inducible cell lines provide insight into p53 responses in tumor cells.

The cell lines described herein have provided several novel observations about the cellular response to p53. We show for the first time that within a given clonal cell line the level of p53 can determine whether cells arrest or die. We also demonstrate that although DNA damage can cooperate with p53 to elicit an apoptotic response, this occurs without detectable alteration in the amount of the p53 protein. Furthermore, our results show that the arrest and apoptotic responses are genetically separable activities of p53. Finally, our data suggest that the p53 protein has multiple domains that function in inducing cell death and that these domains cooperate synergistically to produce a full apoptotic response.

The response of Saos2 cells to the induction of p53 was dictated by the quantities of protein produced. When levels of p53 were lower, cells showed slowed or arrested growth, while at higher levels of p53, cell death ensued. As a transcriptional regulator, p53 binds to its cognate sites in p53 responsive genes and activates transcription of those genes. Both the human and the mouse p21 promoters contain two separated p53 binding sites (El-Diery et al. 1993; Macleod et al. 1995). Looping of DNA mediated through interaction of p53 bound at both sites has been demonstrated to facilitate transactivation by p53 (Wang et al. 1995a). Because of this configuration of cooperating sites, one might predict that p21 would require relatively little p53 for optimal activation. Indeed, our preliminary results suggest that maximal activation of p21 in p53-7 cells is obtained even with very low levels of p53 (data not shown) and thus, very little p53 is required to drive expression of p21 and consequently to effect growth arrest.

The N-terminus of p53 contains a novel activity that regulates p53-dependent cell cycle arrest and apoptosis.

Activation of the endogenous p21 gene by p53 has been used to measure p53 transcriptional activity (Attardi et al. 1996; Chen et al. 1996; Sabbatini et al. 1995; Walker and Levine 1996; Wang et al. 1996). p53(Δ1-42) is capable of activating expression of the endogenous p21 gene in cells while p53(Δ1-63) is completely inert. In addition, the expression of the mdm2 gene, another p53 target, was also activated by p53(Δ1-42) (data not shown). Numerous studies have showed that p53-dependent cell cycle arrest requires its transcriptional activation function (Attardi et al. 1996; Chen et al. 1996; Pietenpol et al. 1994; Sabbatini et al. 1995). Consistent with the observation that p53(Δ1-42) can activate p21 expression, we also showed that p53(Δ1-42) can efficiently induce cell cycle arrest. Together, these results strongly suggest that another potential activation domain lies between residues 43 to 63 in addition to the original defined activation domain within residues 1-42. Within this stretch of 21 residues there are seven acidic, eight hydrophobic and zero basic residues, all of which are characteristics of an acidic activation domain (Mitchell and Tjian. 1989). A second activation domain within a transcriptional factor is not without precedent. Herpes simplex virus (HSV) protein VP16 also contains two independent activation domains (Regier et al. 1993). Since p53(Δ1-42) can induce apoptosis and its capability to induce apoptosis is even greater than that of wild-type p53 (table 1) while p53(Δ1-63) is completely defective in inducing apoptosis (Table 2). Together, these results suggest that an apoptosis promoting domain is located between residues 43 to 63.

Transcriptional activation and apoptosis.

Several reports, including our own, propose that p53 can induce apoptosis in several types of cells without transcriptional activation (Caelles et al. 1994; Chen et al. 1996; Haupt et al. 1995; Wagner et al. 1994). This hypothesis is primarily based on two lines of evidence; first, the ability of p53 to activate transcription is not proportionally correlated to that of p53 to induce apoptosis; second, the transactivation deficient mutant p53(gln22-ser23), which cannot activate expression of the endogenous p21 gene in vivo and consequently cannot induce cell cycle arrest, is still capable of inducing apoptosis. However, a question arises since p53(Δ1-42), which lacks the entire previously defined activation domain, not only induce a strong apoptosis, but also activates expression of the endogenous p21 gene. The transactivation deficient p53(gln22-ser23) protein also contains the intact activation domain located within residues 43-63. Thus, a possibility exists that a distinct class of target genes necessary for apoptosis might be regulated by p53(gln22-ser23) although such a target has yet been identified.

p53 expressing cells are more sensitive to apoptosis when DNA damaged

Even though a low level of p53 is not sufficient to induce apoptosis in Saos-2 cells, it can sensitize cells to undergo apoptosis following CPT-induced DNA damage. Because calcium phosphate-mediated DNA transfection of cells can induce p53 and an ensuing growth arrest (Renzing and Lane 1995), this procedure might also cooperate with expressed p53 to amplify the apoptotic response in transient transfection assays. This may explain why our experiments show a much weaker and delayed cell death for the p53 (gln22/ser23) cell lines than previously reported when transient transfection assays were employed (Haupt et al. 1995). How these two agents, DNA damage and p53, cooperate is a matter of great interest. While there is considerable evidence that p53 is stabilized after DNA damage, it has been speculated that DNA damage might also convert p53 to a more active DNA binding state (Lu and Lane 1993). This intriguing possibility is currently under investigation. We are excited by the potential of using low p53 producer cells to screen a variety of cancer therapy drugs which may cooperate with p53 to induce apoptosis. Thus, it is hoped that more effective chemotherapeutic drugs can eventually be identified.

p53 stability.

We show here that the N-terminal 96 amino acids but not the C-terminal 30 amino acids are required for ubiquintin/calpain-mediated destabilization of p53 and DNA-damage induced stabilization is in part attributable to phosphorylation at serine 15. However, the increased charges as a result of substituting serine at residue 15 to aspartate is not sufficient in stabilizing p53, suggesting that the actual presence of the phosphate group is important in regulating the stability of p53.

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APPENDIX A.

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Publications:

1. **Chen, X.**, J. Bargonetti, and C. Prives. 1995. p53, through p21 (WAF1/CIP1), induces cyclin D1 synthesis. **Cancer Res.** 55:4257-4263.

2. **Chen, X.**, L. J. Ko, L. Jarayaman, and C. Prives. 1996. p53 levels, functional domains and DNA damage determine the extent of the apoptotic response of tumor cells. **Genes & Dev.** 10:2438-2451.

3. Zhu, J., W. Zhou, and **X. Chen.** 1997. Identification of novel p53 functional domains that regulate p53-dependent cell cycle arrest and apoptosis. Submitted to MCB.

Meeting Abstracts:

1. Chen, X., and C. Prives. p53, through p21 (waf1/cip1), induces a novel form of cyclin D1 presented at Cancer genetics and tumor suppressor genes meeting held at Frederick, Maryland. June 14-17, 1995.
2. Chen, X., and C. Prives. A full apoptosis response requires both sequence-specific transactivation and carboxyl terminal regulatory domain of p53 presented at Cancer genetics and tumor suppressor genes meeting held at Cold Spring Harbor, New York. August 14-18, 1996.
3. Zhu, J., and X. Chen. Delineation of domains of p53 required for p53-dependent cell cycle arrest and apoptosis presented at Cancer genetics and tumor suppressor genes meeting held at Frederick, Maryland. June 12-16, 1997.

Personnel receiving pay from the grant support

Xinbin Chen, the Principal Investigator

Sheau-Yann Shieh, the substitute Principal Investigator for the third year of the grant

APPENDIX B.

MATERIALS AND METHODS

Plasmids. The pUHD15-1 neo plasmid contains the tTA transactivator gene derived from pUHD15-1 and the neomycin gene as described in Resnitzky et al. (1994). Genes of interest were cloned into the 10-3 plasmid to allow their conditional expression as described in Gossen and Bujard, (1992). The pBabe plasmid contains the puromycin resistance gene (Morgenstein and Land, 1990) and was used for secondary selection as described below. To construct tetracycline regulated expression vectors, the following cDNA fragments were cloned into the 10-3 vector: wild-type p53 (Baker er al. 1990), tumor-derived mutant forms of p53 (his 175 and ser 249) (Baker et al. 1990), the transactivation-deficient form of p53 (gln22/ser23) (Lin et al., 1994), deletion mutants lacking either N-terminal 22 (Δ1-22), 96 (Δ1-96) or C-terminal 30 (Δ364-393) amino acids (Jayaraman and Prives, unpublished), a transactivation defective form of p53 which lacks the C-terminal 30 amino acids (gln22/ser23Δ364-393) (see below for construct generation), or p21 (WAF1) (El-Deiry et al. 1993). To generate p53 (gln22/ser23Δ364-393), the C-terminus of the p53 (gln22/ser23) cDNA beginning at amino acid 144 at the PvuII site was replaced by the C-terminus of the p53(Δ364-393) cDNA.

Mutagenesis. The p53 deletion mutants were generated by PCR to amplify the corresponding sequences using the full-length wild-type p53 cDNA as a template. Mutations were confirmed by sequencing. All proteins are tagged at their N-termini with the influenza hemaglutinin (HA) peptide. The primers used to generate p53(Δ1-23) are; forward primer GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TAA ACT ACT TCC TGA A; universal reverse primer GAT CGA ATT CTC AGT CTG AGT CAG GCC CTT. The primers for p53(Δ1-42) are forward primer GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TTT GAT GCT GTC CCC G plus the universal reverse primer. The primers for p53(Δ1-63) are forward primer GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TCC CAG AAT GCC AGA GGC T plus the universal reverse primer.

Cell lines. The Saos-2, T98G and H1299 cells were purchased from the American Type Culture Collection. RKO cells were obtained from M. B. Kastan (Kastan et al. 1992). 10(1) cells were obtained from A. J. Levine (Harvey and Levine 1991). 3-4 cells were generated by transfection of 10(1) cells with the temperature-sensitive murine p53val135. GM47-23 and Del4A cells were obtained from W. E. Mercer (Mercer et al., 1990a; 1990b; Lin et al., 1992a). All cells were grown in Dulbocco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO2.

Transfection and selection procedures Transfections were performed using the calcium chloride method as described (Chen and Okayama, 1987). To generate cell lines expressing inducible proteins of interest, a two step procedure described by Resnitzky et al. (1994) was used as summarized below. First, low passage Saos-2 or H1299 cells were transfected with pUHD15-1 neo and clones were selected and maintained in the presence of 400 and 250 μg/ml of active G418 (Geneticin, Gibco), respectively. To test for cloned cell lines capable of inducing expression from the tetracycline promoter, the 10-3(p53) plasmid was transiently transfected into those cells in the presence of tetracycline (1 μg/ml) for 10-18 h and the transfected cells were then split 1:2 and grown in the presence or absence of tetracycline. Cells were extracted 24-48 h later, and expression of the p53 protein was determined by Western blot analysis. Two of the 15 clonal Saos-2 cell

lines (S32 and S2) and two of the 30 clonal H1299 cell lines (H15 and H24) were found to induce p53 expression upon withdrawl of tetracycline. Since p53 is a very potent suppressor of cell growth, cell lines with "leaky" expression of p53 could not be stably propagated. To ascertain that these cells do not induce expression from the tetracycline promoter in the presence of tetracycline, they were cotransfected by 10-3(p53) and a plasmid containing a luciferase reporter gene under control of the gadd45 p53 responsive element (gadd45-luc) (Chen et al. 1995). The S32 and H24 cell lines were found to induce lower levels of basal luciferase activity in the presence of tetracycline than S2 and H24 cell lines, respectively. In addition, the fold of induction of luciferase activity in the absence of tetracycline is two times higher in S32 and H24 cells than in S2 and H15 cells, respectively. Therefore, both S32 and H24 cell lines were used as parental cell lines for subsequent generation of inducible cell lines. Second, various 10-3 plasmids containing cDNAs encoding either wild type, or mutant forms of p53 or p21 were cotransfected with the puromycin selectable pBabe plasmid (Morgenstern and Land 1990) into either S32 or H24 cells. Clones were selected and maintained in the presence of 2 μg and 1 μg of puromycin (Sigma) per ml, respectively. Individual clones were screened for inducible expression of the p53 and p21 proteins by Western blot analysis using monoclonal antibodies against p53 and p21 as described below.

Antibodies. The various monoclonal antibodies used to detect p53 were PAb1801 and PAb421 as described (Chen et al., 1995). The affinity-purified monoclonal antibody against p21 (Ab-1), Bcl-2 (Ab-1) was purchased from Oncogene Science (Uniondale, NY) and affinity-purified monoclonal antibodies against Bax (P-19) and anti-actin polyclonal antibodies was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Sigma (St. Louis, MO), respectively. Affinity-purified monoclonal antibodies against PCNA, cyclin B1 and cyclin D1 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Affinity-purified polyclonal antibodies against D-type cyclins were purchased from Upstate biotechnology Incorporated [cyclin D1 (UBI)] (Lake Placid, NY), and Santa Cruz Biotechnology Inc. (cyclin D2 and D3) (Santa Cruz, CA). Rabbit polyclonal antisera against CDK4, CDK2 and cdc2 were kindly provided by Z.-Q. Pan (Pan and Hurwitz 1993). Anti-cyclin A, C160, was a mouse monoclonal antibody (Whyte et al. 1988) raised against the cyclin A protein that associated with adenovirus E1A protein. PAb 419 is a mouse monoclonal antibody against SV40 large T antigen (Harlow et al., 1981). To generate anti-p21 antibody, a 900-bp StuI-EcoRI cDNA fragment encoding a.a. 17 to 164 of p21 polypeptide (El-Deiry et al. 1993) was inserted in-frame into pRSETB expression vector (Invitrogen). The his-tagged p21 protein was then expressed in bateria and purified from Ni-agarose beads and anti-p21 antibody was raised in a rabbit. pcDNA3-p21 was generated by inserting a 1.0kb EcoRI-EcoRI fragment of p21 cDNA (El-Deiry et al. 1993) into pcDNA3 (Invitrogen).

Immunoblot analysis. Cells were collected from plates in phosphate-buffered saline (PBS) and resuspended with 1x sample buffer, and boiled for 5 min. For immunoblot analysis, a standard procedure was followed as previously described (Chen et al. 1995).

Immunoprecipitation. Cells were grown in DMEM (methionine minus) media plus 10% of fetal bovine serum for 1 h and labeled with 75 μCi/ml of Tran^{35}S-label methionine (ICN Pharmacerticals) for 1 h. Whole cell extracts were prepared by lysing cells with the NP-40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 7.5), 1 mM DTT, 0.5% of NP-40, 25 ng/ml of aprotinin, 25 ng/ml of leupeptin, and 1 mM PMSF] for 15 min on ice. The extracts were precleared with 10 μl of normal rabbit serum plus 100 μl of 10% *Staphylococcus aureus* cells (The Enzyme Center, Malden, MA) for 1 h at 4oC and clarified by centrifugation. Immunoprecipitations were performed by the addition of 10 μl of various antisera as described in the Figure Legends and 40 μl of 50% protein A

sepharose (Pharmacia, Piscataway, NJ). The immunoprecipitates were washed three times with the NP-40 lysis buffer prior to the addition of 2x sample buffer and boiling, followed by SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography.

Growth rate analysis. To determine the rate of cell growth, 5-10 $\times$ 10^4 cells were seeded per 60-mm plate with or without tetracycline. The medium was replaced with fresh medium with or without tetracycline every 72 h. At indicated times, two plates were rinsed with PBS twice to remove dead cells and debris, and live cells on the plates were trypsinized and collected individually. Cells from each plate were counted by Coulter cell counter three times. The average number of cells from at least two plates were used for growth rate determination.

FACS analysis. 2.0 $\times$ 10^5 cells were seeded per 90-mm plate with or without tetracycline. Three days after plating, both floating dead cells in the medium and live cells on the plates were collected and fixed with 2 ml of 70 % ethanol for at least 30 min. For FACS analysis, the fixed cells were centrifuged and resuspended in 1 ml of PBS solution containing 50 µg/ml each of RNase A (Sigma) and propidium iodide (PI) (Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter (FACSCaliber, Becton Dickinson) within 4 hours. The percentage of cells in sub-G_1, G_0-G_1, S, and G_2-M phases was determined by using the ModFit program. The percentage of cells in sub-G_1 phase was used as an index for the degree of apoptosis.

Cell viability assay by trypan blue exclusion. 2 $\times$ 10^5 cells were seeded per 90-mm plate with or without tetracycline. 3 days after plating, both floating cells in the culture medium and cells on the plate were collected and concentrated by centrifugation. After stained with trypan blue (Sigma) for 15 min, both live (unstained) and dead (stained) cells were counted in a hemocytometer twice. After subtraction of the percentage of dead cells from the control plates, the percentage of dead cells from the experimental plates was used as an index for the degree of apoptosis. After several side by side comparisons, we found that the percentage of dead cells determined by trypan blue exclusion assay is comparable to, or slightly lower than, that determined by FACS analysis above.

RNA isolation and Northern blot analysis. Poly(A)+RNA was isolated by using the QuickPrep mRNA purification kit (Pharmacia) as described. Northern blot analysis was performed as described (Chen and Velicer 1990). The cyclin D1 probe was made from a 1.0-kb NcoI-HindIII cDNA fragment (Xiong et al. 1991). The GAPDH probe was made from a 1.25-kb PstI-PstI cDNA fragment (Marty et al. 1985). The p21 probe was made from a 1.0-kb EcoRI-EcoRI cDNA fragment (El Deiry et al., 1993).

APPENDIX C

Figure Legends

Fig. 2. p53-dependent transactivation of p21 in GM47-23 cells. GM47-23 cells were ^{35}S-methionine labeled in the presence or absence of dexamathasone treatment (24 h) as described (Mercer et al., 1990a). The ^{35}S-labeled extracts were immunoprecipitated with a control antibody PAb419 (lanes 1-2), anti-p53 antibodies PAb1801 (lanes 3-4) and PAb122 (lanes 5-6), and anti-p21 antibody (lanes 7-8). Dexamathasone treatment is indicated by + or -, respectively, at the top of each lane. Proteins whose identities have been established are marked at right, and molecular weight markers are indicated at left.

Fig. 3. Kinetics of p53-dependent cyclin D1 induction. (A) GM47-23 cells treated with dexamathasone for 0-48 h as indicated at the top of each lane were ^{35}S-methionine labeled. The labeled cell extracts were subject to immunoprecipitation with antibodies against p21 (lanes 1-6) and cyclin D1 (lanes 7-12). The cyclin D1, CDKs and p21 proteins are marked at right. (B) An immunoblot was prepared using extracts from GM47-23 cells treated with dexamathasone for 0-36 as indicated at the top of each lane. The blot was probed with anti-p53 monoclonal antibody PAb1801. The inducible and endogenous p53 proteins are indicated at right. (C) Mouse 3-4 cells (lanes 1-6) grown at 32°C for 0-48 h as indicated at the top of each lane, were ^{35}S-methionine labeled. The labeled cell extracts were subject to immunoprecipitation with anti cyclin D1 antibody. The cyclin D1 protein is indicated at right. (D) 10(1) cells (lanes 1-4) grown at 32°C for 0-24 h as indicated at the top of each lane, were ^{35}S-methionine labeled. The labeled cell extracts were subject to immunoprecipitation with anti-cyclin D1 antibody. The cyclin D1 protein is indicated at right.

Fig. 4. p53-dependent induction of cyclin D1 synthesis. (A) GM47 23 cells treated with dexamathasone for 0 or 24 h were ^{35}S-methionine labeled. The labeled cell extracts were subject to immunoprecipitation with antibodies against cyclin D1 (lanes 1-2) and p21 (lanes 3-4) as indicated at the top of each lane. Proteins whose identities have been identified are marked at right. (B) and (C) T98G cells (B) or Del4A cells (C) treated with dexamathasone for 0 or 24 h were ^{35}S-methionine labeled. The labeled cell extracts were subject to immunoprecipitation with antibodies against p53 (PAb1801) (lanes 1-2), p21 (lanes 3-4), and cyclin D1 (lanes 5-6). The cyclin D1, and inducible and endogenous mutant p53 proteins are indicated at right.

Fig. 5. p53-dependent induction of cyclin D1 synthesis. (A) GM47-23 cells treated with dexamathasone for 0 or 24 h were ^{35}S-methionine labeled. The labeled cell extracts were subject to immunoprecipitation with antibodies against CDK4 (lanes 1-2), CDK2 (lanes 3-4), cdc2 (lanes 5-6), cyclin B1 (lanes 7-8), and PCNA (lanes 9-10) as indicated at the top of each lane. Proteins whose identities have been identified are marked at right. (B) An immunoblot was prepared using extracts from GM47-23 cells treated with dexamathasone for 0-36 as indicated at the top of each lane. The blot was sequentially probed with anti-cyclin A monoclonal antibody (C-160) (top panel) and rabbit anti-actin polyclonal antibody (bottom panel). The cyclin A and actin proteins are indicated at right.

Fig. 6. DNA damage triggered p53-dependent cyclin D1 induction. ^{35}S-labeled extracts from RKO cells untreated or treated with camptothecin were subjected to immunoprecipitation with anti-p53 antibody PAb1801 (A) or anti-cyclin D1 (B, lanes 1-2) and anti-p21 (B, lanes 3-4)) as indicated at the top of each lane. The p53, cyclin D1 and p21 proteins are indicated at right.

Fig. 7. p53-dependent transcriptional induction of cyclin D1. A Northern blot was prepared using 0.5 μg of poly(A)+ RNA isolated either from GM47-23 cells (lanes 1-6) treated with dexamathasone for 0-48 or from T98G cells (lanes 7-11) treated with dexamathasone for 0-24 h. The blot was probed sequentially with cyclin D1 DNA (top panel), GAPDH DNA (middle

panel), and p21 DNA (bottom panel). The identities of cyclin D1, GAPDH, and p21 mRNAs are marked at right.

Fig. 8. p21 at least partially mediates p53-dependent induction of cyclin D1. Subconfluent T98G cells were transiently transfected with 0 μg (lanes 1 and 5), 5 μg (lanes 2 and 6), 15 μg (lanes 3 and 7), 30 μg (lanes 4 and 8) of pcDNA3-p21 DNA and the transfected cells were ^{35}S-methionine labeled. The labeled extracts were subject to immunoprecipitation with anti-cyclin D1 (lanes 1-4) or anti-p21 (lanes 5 8) antibodies. The identities of cyclin D1, p21 and CDKs are marked at right.

Fig. 9. p53 preferentially induces expression of the rapidly migrating cyclin D1. (A) Immunoblots were prepared using extracts from GM47-23 cells treated with dexamathasone for 0-36 h. The blots were probed with anti-cyclin D1 (top and middle panels) and anti-actin (bottom panel) as indicated at left of each panel. (B) Immunoblots were prepared using extracts from mouse 3-4 cells grown at 32°C for 0-48 h. The blots were probed with anti-cyclin D1 (top and middle panels) and anti-actin (bottom panel) as indicated at left of each panel. The cyclin D1 and actin proteins are marked at right of each panel.

Fig. 10. The level of p53 determines cell death or arrest. (A) Growth rates of p53-7 cells in the presence or absence of tetracycline. Note that in this and other experiments there was a slight decrease in cell number at day 1 which is most likely due to trypsinization. (B) p53-7 cells were grown in the presence of 1,000, or 0 ng/ml of tetracycline for 1, 2 and 3 days, and the % of cells in each phase of cell cycle were determined by flow cytometry. (C) Inducible expression of p53, and levels of actin in p53-7 cells in the presence of 1,000, 40, 20, 10, 8, 6, 4, 2, 1, and 0 ng/ml of tetracycline as indicated were assayed by western blot analysis. The blots were probed with p53 monoclonal PAb1801 and actin polyclonal antibodies, respectively. (D) The growth rates of p53-7 cells in the presence of varying concentrations of tetracycline were measured.

Fig. 13. Deletion of the N-terminal 22 amino acids enhances the ability of p53 in inducing apoptosis. (A) Inducible expression of p53, p21 and levels of actin in p53(Δ1-22)-2, -3, -4 and -5 cell lines in the presence or absence of tetracycline (2 μg/ml) for 24 hr were assayed by Western blot analysis. The top portion of the blot was probed with a mixture of p53 monoclonal antibodies PAb421, PAb240 and PAb1801, and actin polyclonal antibody. The bottom portion of the blot was probed with p21 monoclonal antibody. (B) Growth rates of p53(Δ1-22)-3 cells in the presence or absence of tetracycline. (C) 2×10^5 p53(Δ1-22)-3 cells were seeded per 10-cm plate in the presence or absence of tetracycline for 3 days and percentage of cells in the sub-G_1, G_0-G_1, S and G_2-M phases were quantitated by FACS analysis.

Fig. 14. Residue 23 is important for p53 activity in transactivation and apoptosis. The experiments were performed in an identical manner to those in Fig. 13.

Fig. 15. p53(Δ1-42), which lacks the entire activation domain, is capable of activating transcription and mediating cell cycle arrest and apoptosis. The experiments were performed in an identical manner to those in Fig. 13.

Fig. 16. The N-terminal 63 amino acids are required for p53 to activate transcription and mediating cell cycle arrest and apoptosis. The experiments were performed in an identical manner to those in Fig. 13.

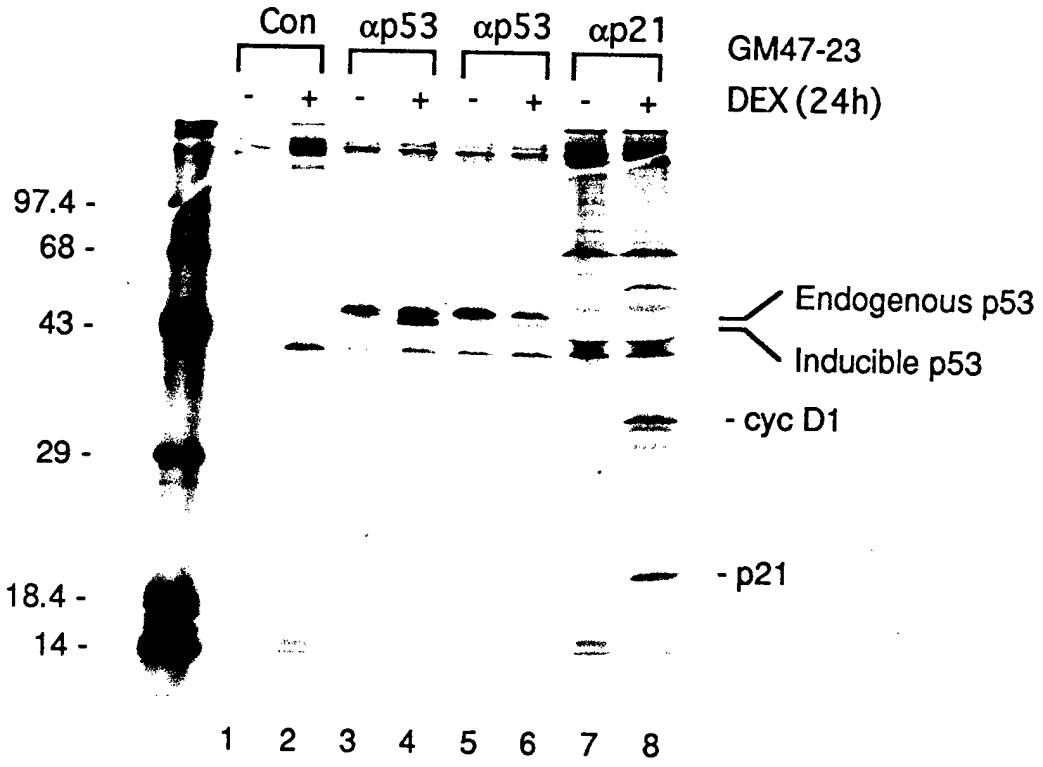
Fig. 17. N-terminal 96 amino acids but not the C-terminal 30 amino acids are required for ubiquitin/calpain-mediated destabilization of p53. (A) Stabilization of endogenous and transfected,

HA-tagged p53 by the calpain/proteasome inhibitor LLnL. LNCaP cells were transfected with HA-tagged full-length p53, and treated with 50 μM LLnL 2 hours before extracts were prepared. p53 was detected with Western using a cocktail of p53-specific antibody. (B) The N-terminal 96 amino acids are essential, while the C-terminal 30 amino acids are dispensable for calpain/proteasome mediated degradation of p53. Cells were transfected with HA-tagged truncated p53 as indicated and expressed proteins were immunoprecipitated with 12CA5 antibody and detected with Western using the same antibody.

Fig. 18. Phosphorylation at serine 15 contributes to stabilization of p53 following DNA damage. (A) Phosphorylation at serine 15 is induced by various DNA damaging agents. LNCaP cells were treated with either DMSO (solvent), γ irradiation (7G), camptothecin (CPT, 0.25 μM), Actinomycin D (Act D, 5 nM), or UV irradiation (50 JM^{-2}), then harvested 2 hours later (CPT, γ) or the next day (Act D, UV). p53 was immunoprecipitated and detected by Western using either the p53 antibodies (PAb421, PAb1801) or rabbit anti phospho-Ser15 antiserum (PS15). With higher concentration of actinomycin D, phosphorylation at serine 15 was also observed. (B) Phosphorylation at serine 15 was not observed in p53 stabilized by LLnL. (C) Induction of phosphorylation at serine 15 coincides with stabilization of 53 upon γ-irradiation in LNCaP cells. Lanes 7 and 8 contains baculovirus-expressed purified p53 that had been either treated with alkaline phosphatase (lane 7) or phosphorylated in vitro with purified DNA-PK. (D) Stabilization of 53 by the DNA damaging agent CPT is hindered in the presence of the PI-3 kinase-specific inhibitor wortmannin (wort) in human LNCaP cells or in murine CB17 cells.

Fig. 19. Increased negative charges at serine 15 is sufficient in deterring calpain /proteasome-mediated degradation of p53. HA-tagged Δ30 or a mutant with serine 15 mutated to aspartic acid was transfected into LNCaP cells. Cells were either treated with DMSO (-) or with 50 μM LLnL for two hours. Expressed HA-tagged proteins were then immunoprecipitated with 12CA5 antibody and detected with Western using the same antibody.

Figure 2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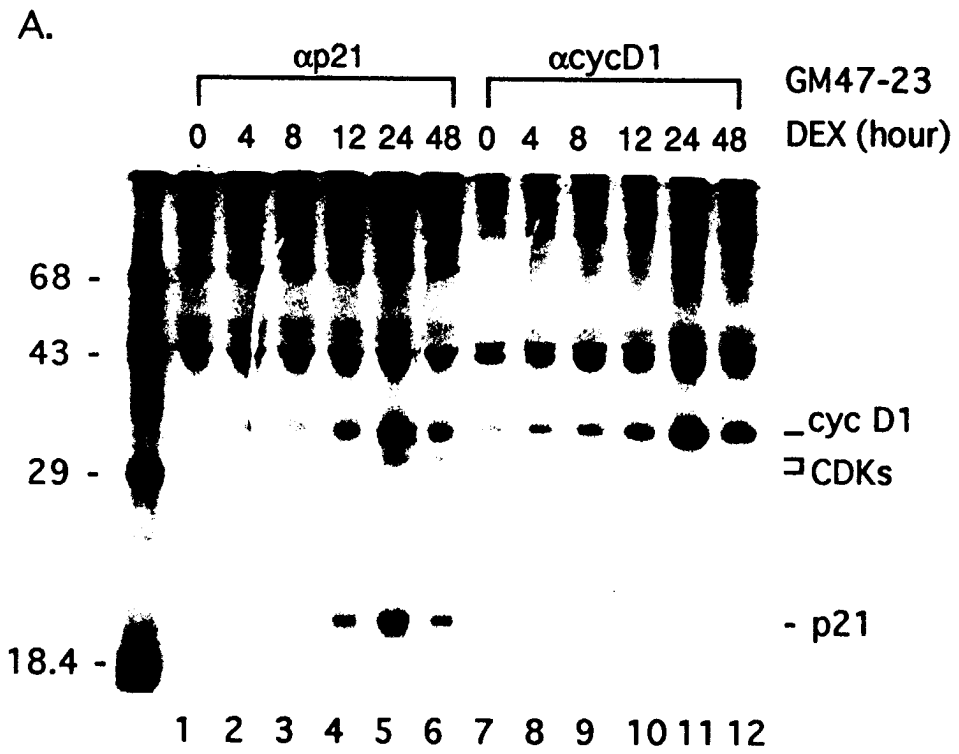
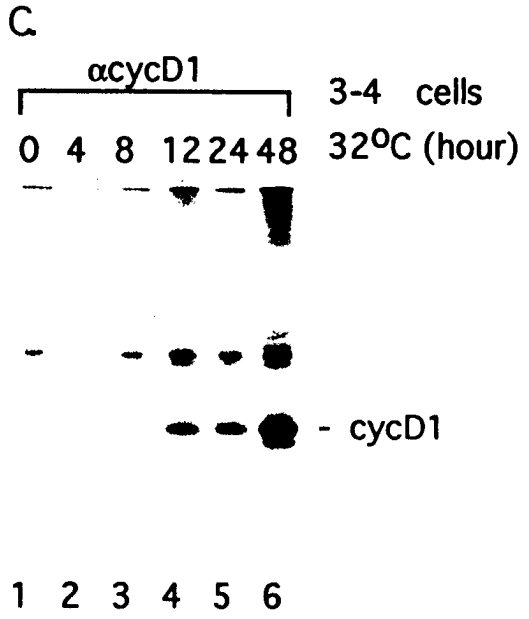
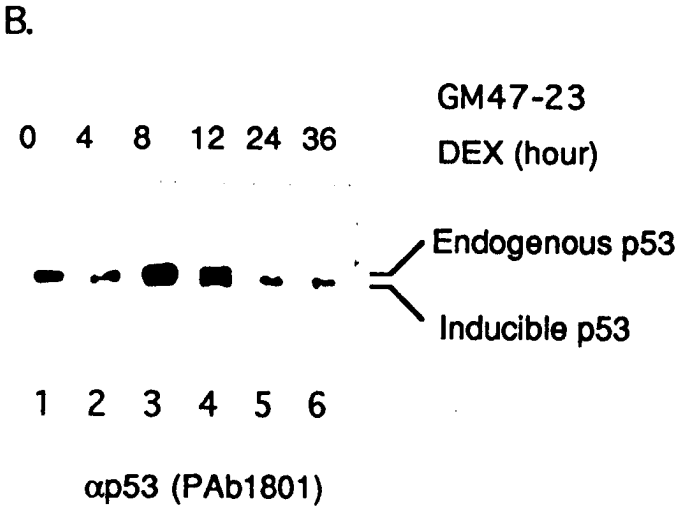
αp53
αp53
αp21
GM47-23
DEX (24h)
- + - + - + - +
97.4 -
68 -
43 -
29 -
18.4 -
14 -
Endogenous p53
Inducible p53
- cyc D1
- p21
1 2 3 4 5 6 7 8

Figure 3


A.
αp21
αcycD1
GM47-23
0 4 8 12 24 48 0 4 8 12 24 48
DEX (hour)
68 -
43 -
29 -
18.4 -
cyc D1
CDKs
p21
1 2 3 4 5 6 7 8 9 10 11 12


C.
αcycD1
3-4 cells
0 4 8 12 24 48
32°C (hour)
cycD1
1 2 3 4 5 6


B.
GM47-23
0 4 8 12 24 36
DEX (hour)
Endogenous p53
Inducible p53
1 2 3 4 5 6
αp53 (PAb1801)

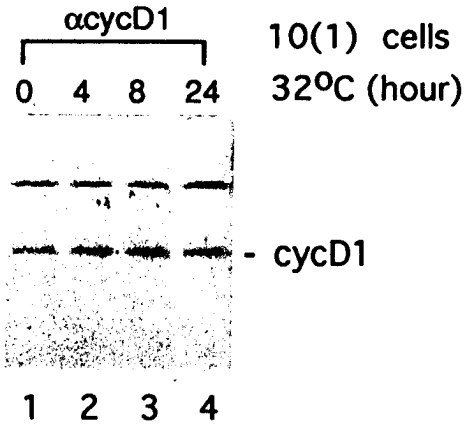

D.
αcycD1
10(1) cells
0 4 8 24
32°C (hour)
cycD1
1 2 3 4

Figure 4

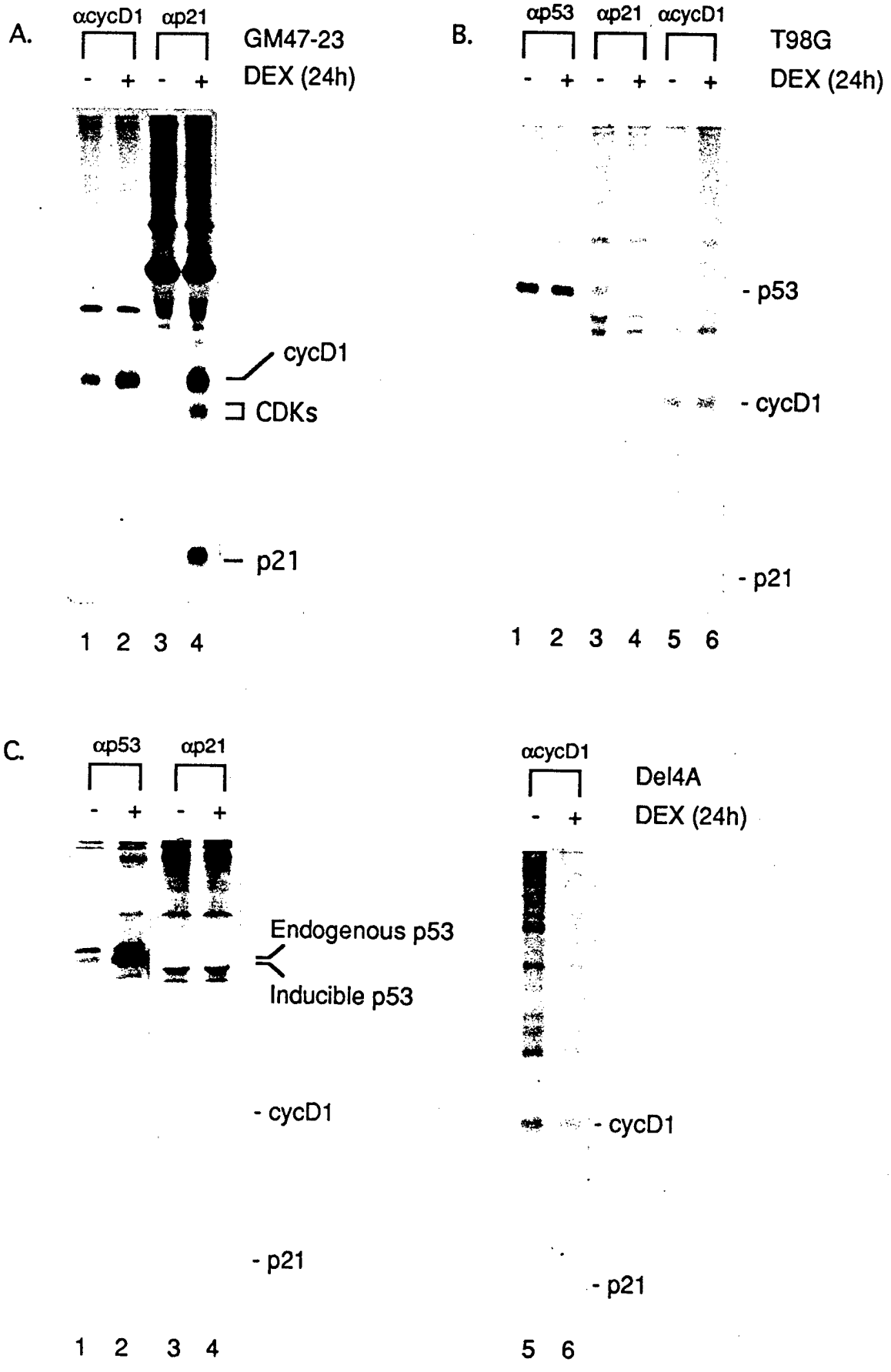
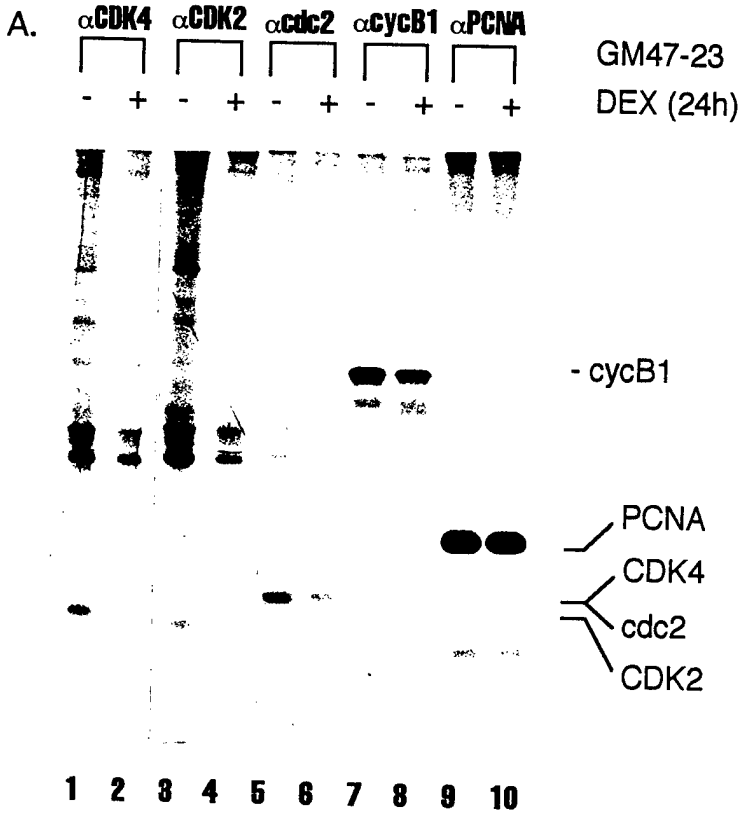

A.
αcycD1
αp21
GM47-23
- + - +
DEX (24h)
cycD1
CDKs
p21
1 2 3 4
B.
αp53
αp21
αcycD1
T98G
- + - + - +
DEX (24h)
- p53
- cycD1
- p21
1 2 3 4 5 6
C.
αp53
αp21
- + - +
Endogenous p53
Inducible p53
- cycD1
- p21
1 2 3 4
αcycD1
Del4A
- +
DEX (24h)
- cycD1
- p21
5 6

Figure 5


A.
αCDK4
αCDK2
αcdc2
αcycB1
αPCNA
GM47-23
DEX (24h)
- + - + - + - + - +
- cycB1
PCNA
CDK4
cdc2
CDK2
1 2 3 4 5 6 7 8 9 10

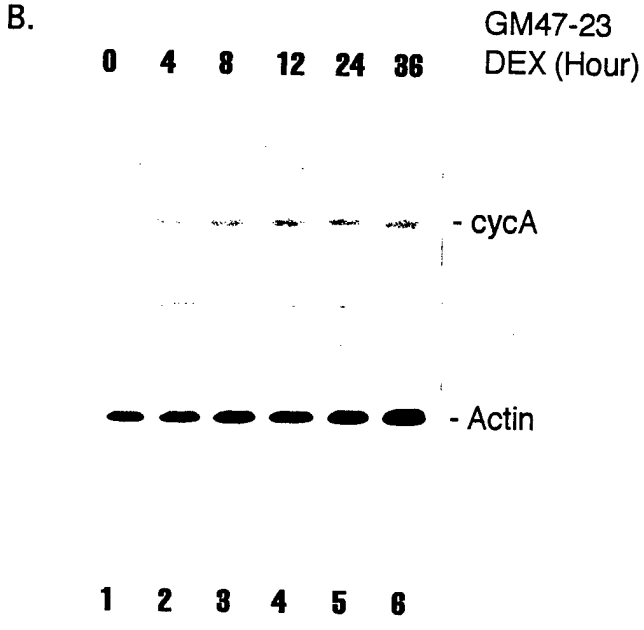

B.
GM47-23
0 4 8 12 24 36
DEX (Hour)
- cycA
- Actin
1 2 3 4 5 6

Figure 6

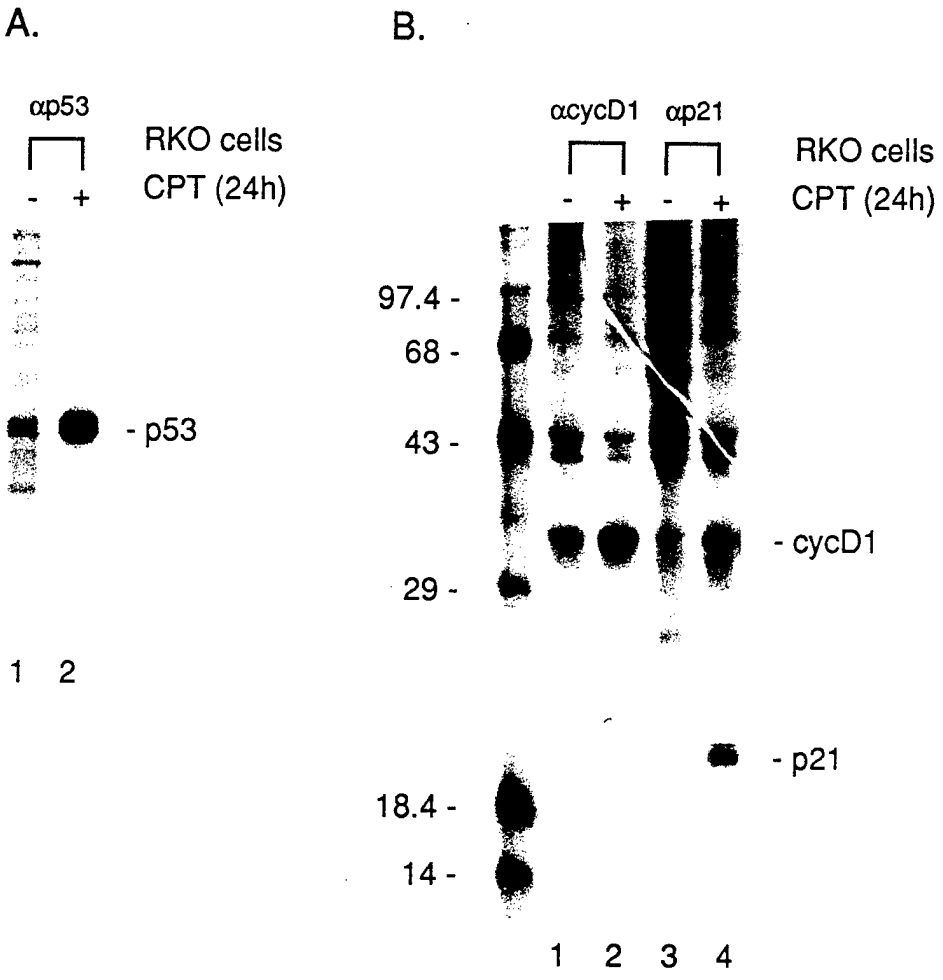

A.
αp53
RKO cells
CPT (24h)
- +
- p53
1 2
B.
αcycD1 αp21
RKO cells
CPT (24h)
- + - +
97.4 -
68 -
43 -
29 -
- cycD1
- p21
18.4 -
14 -
1 2 3 4

Figure 7

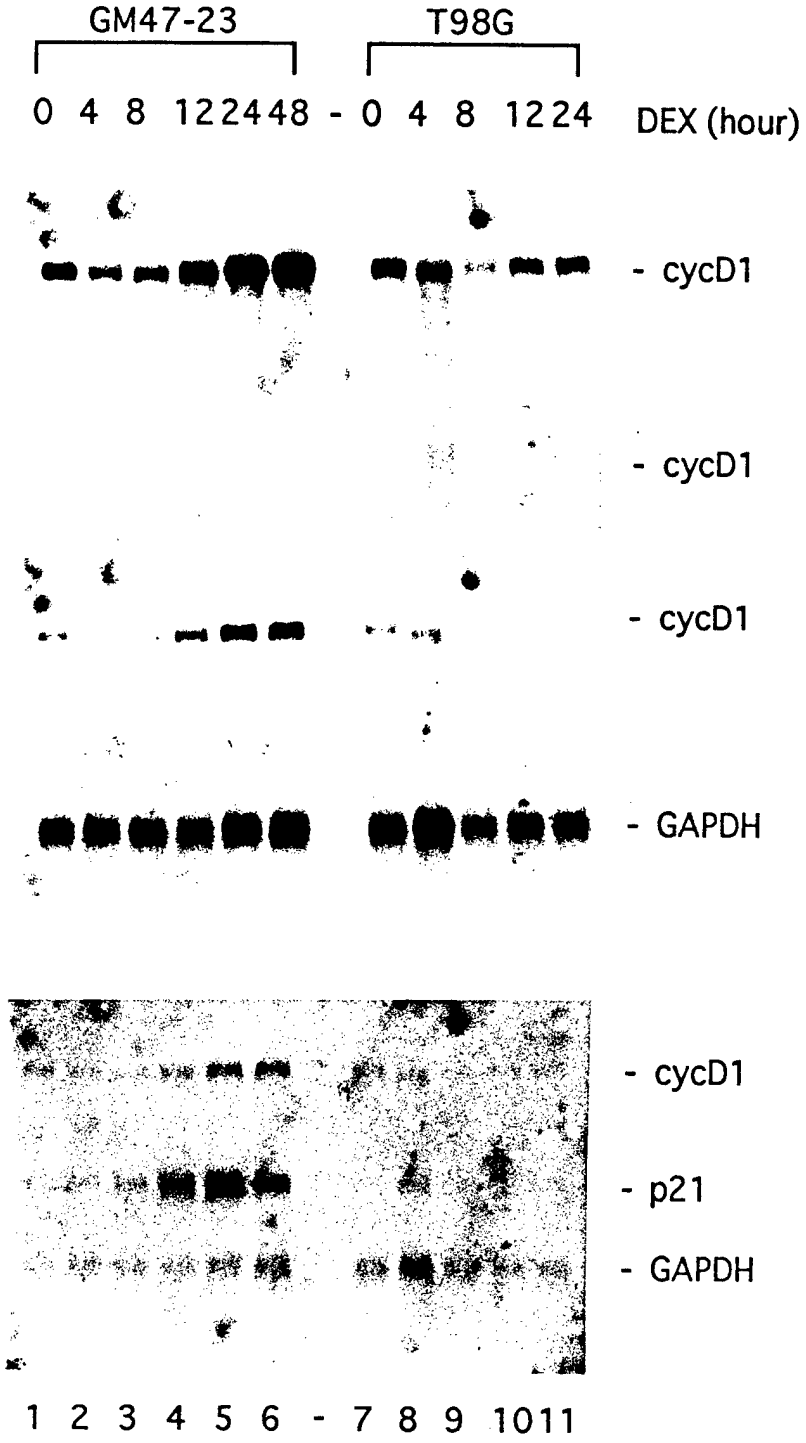

GM47-23
T98G
0 4 8 12 24 48 - 0 4 8 12 24
DEX (hour)
- cycD1
- cycD1
- cycD1
- GAPDH
- cycD1
- p21
- GAPDH
1 2 3 4 5 6 - 7 8 9 10 11

Figure 8

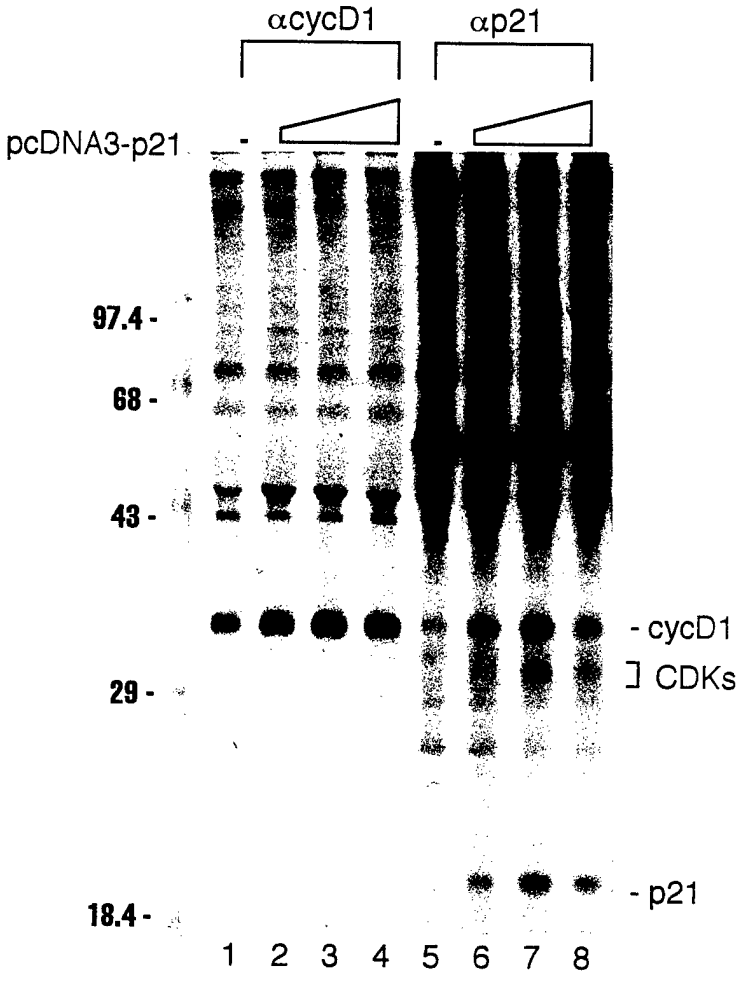

αcycD1
αp21
pcDNA3-p21
-
-
97.4 -
68 -
43 -
29 -
18.4 -
- cycD1
] CDKs
- p21
1 2 3 4 5 6 7 8

Figure 9

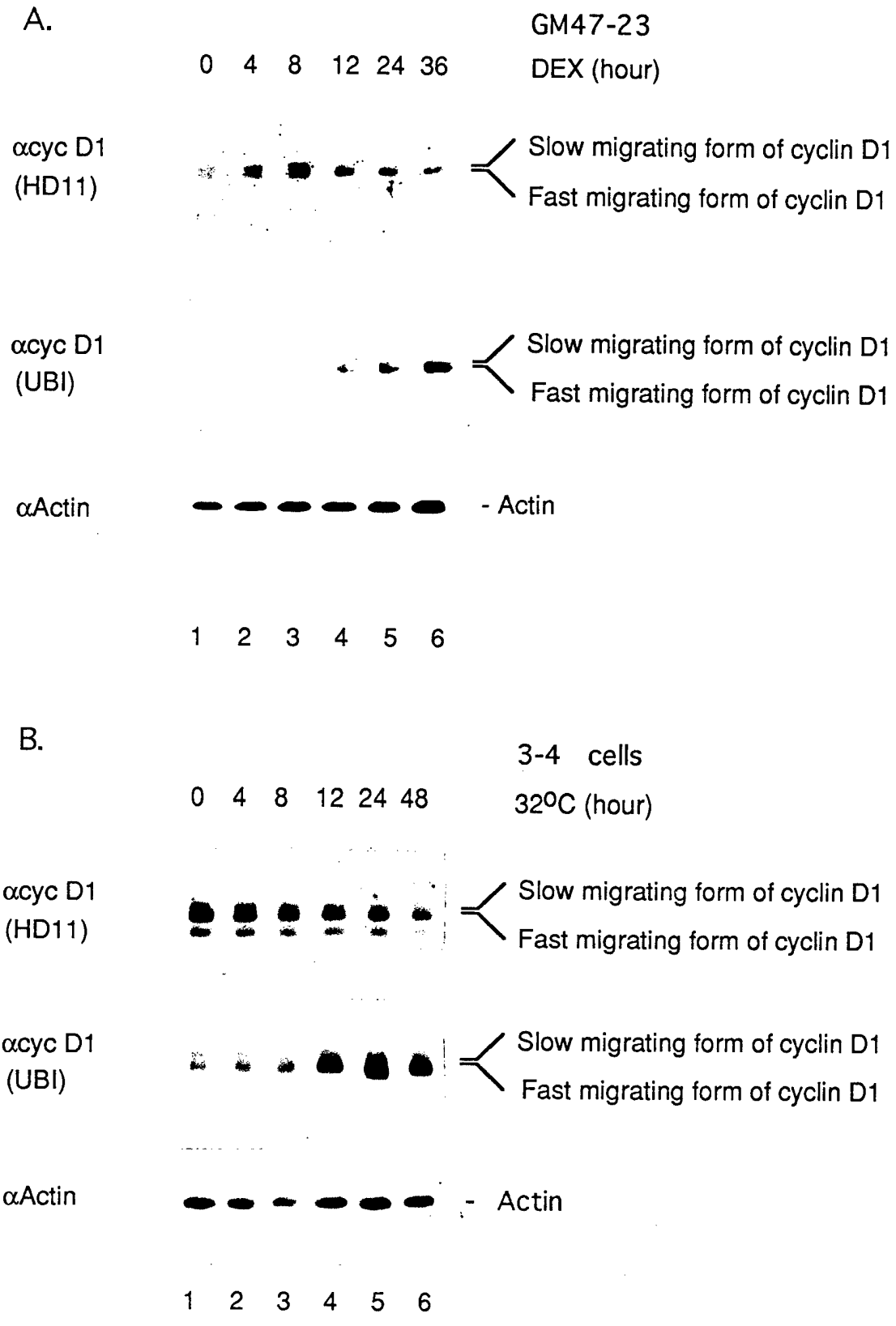
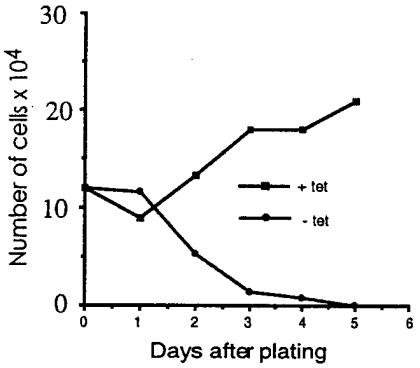

A.
GM47-23
0 4 8 12 24 36
DEX (hour)
αcyc D1
(HD11)
Slow migrating form of cyclin D1
Fast migrating form of cyclin D1
αcyc D1
(UBI)
Slow migrating form of cyclin D1
Fast migrating form of cyclin D1
αActin
- Actin
1 2 3 4 5 6
B.
3-4 cells
0 4 8 12 24 48
32°C (hour)
αcyc D1
(HD11)
Slow migrating form of cyclin D1
Fast migrating form of cyclin D1
αcyc D1
(UBI)
Slow migrating form of cyclin D1
Fast migrating form of cyclin D1
αActin
- Actin
1 2 3 4 5 6

Figure 10

A

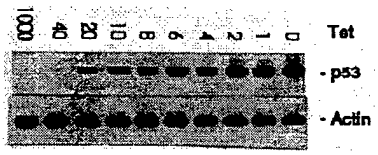


B

FACS analysis of p53-7 cells

Cell cycle phases	% cells in each phase			
	+ tet	- tet/1 day	- tet/2 day	- tet/3 day
G_0-G_1	71.60	72.38	47.85	36.11
S	14.03	4.94	7.28	0
G_2-M	14.37	22.68	19.94	3.46
Sub-G_1	0	0	24.92	60.43

C



D

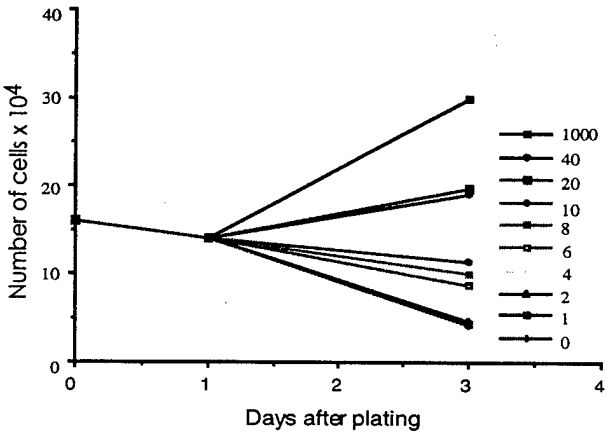


Figure 13

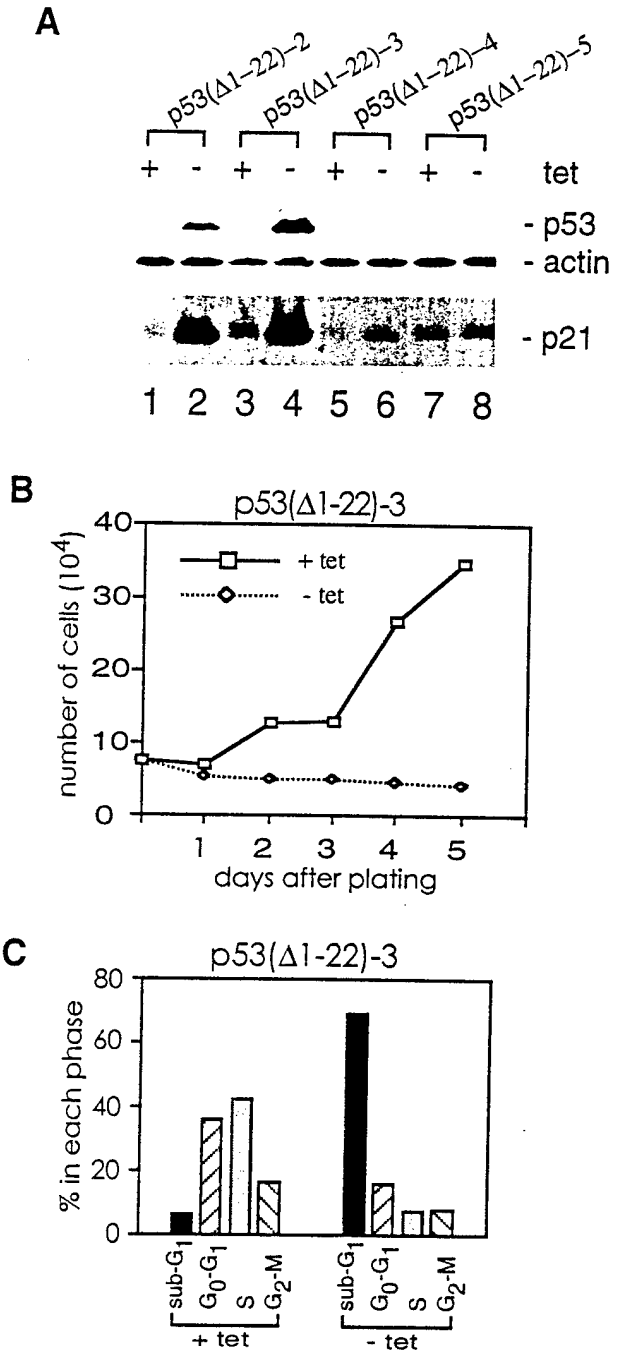

A
p53(Δ1–22)–2
p53(Δ1–22)–3
p53(Δ1–22)–4
p53(Δ1–22)–5
+ - + - + - + -
tet
- p53
- actin
- p21
1 2 3 4 5 6 7 8
B
p53(Δ1-22)-3
number of cells (10^4)
40
30
20
10
0
+ tet
- tet
1 2 3 4 5
days after plating
C
p53(Δ1-22)-3
% in each phase
80
60
40
20
0
sub-G1
G0-G1
S
G2-M
+ tet
- tet

Figure 14

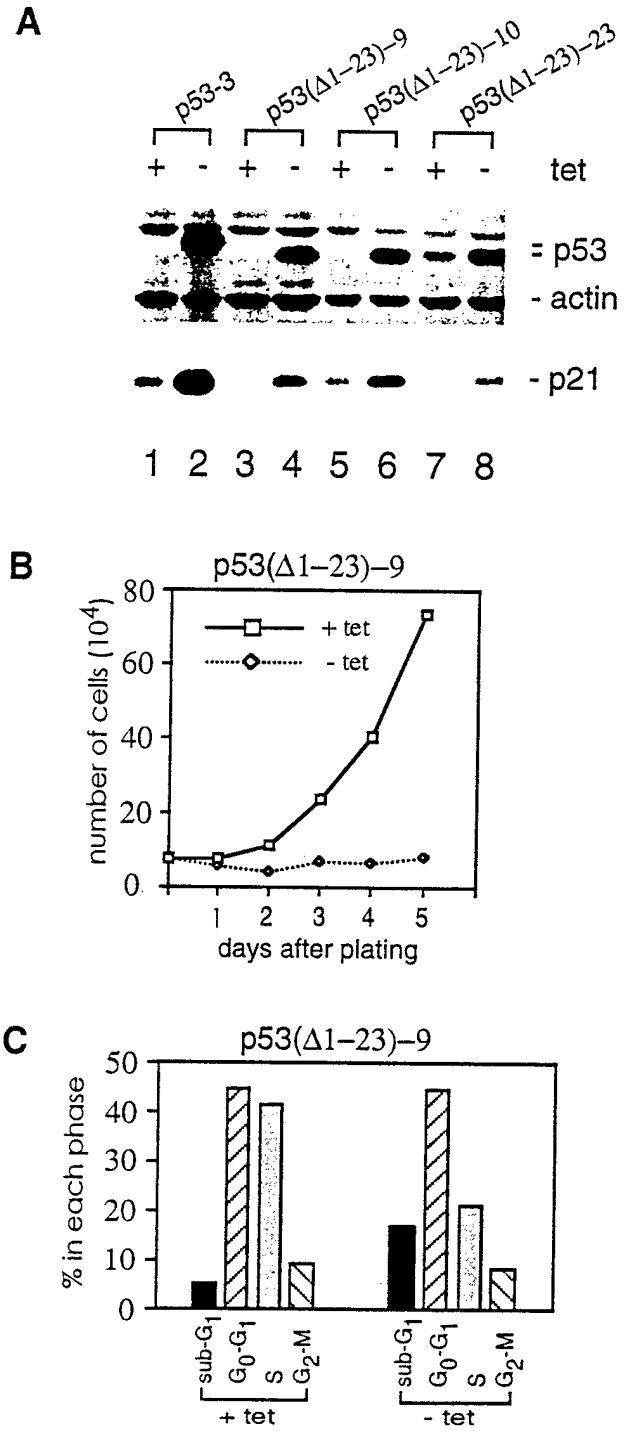

A
p53-3
p53(Δ1–23)–9
p53(Δ1–23)–10
p53(Δ1–23)–23
+ - + - + - + -
tet
= p53
- actin
- p21
1 2 3 4 5 6 7 8
B
p53(Δ1–23)–9
number of cells (10^4)
80
60
40
20
0
+ tet
- tet
1 2 3 4 5
days after plating
C
p53(Δ1–23)–9
% in each phase
50
40
30
20
10
0
sub-G1
G0-G1
S
G2-M
+ tet
- tet

Figure 15

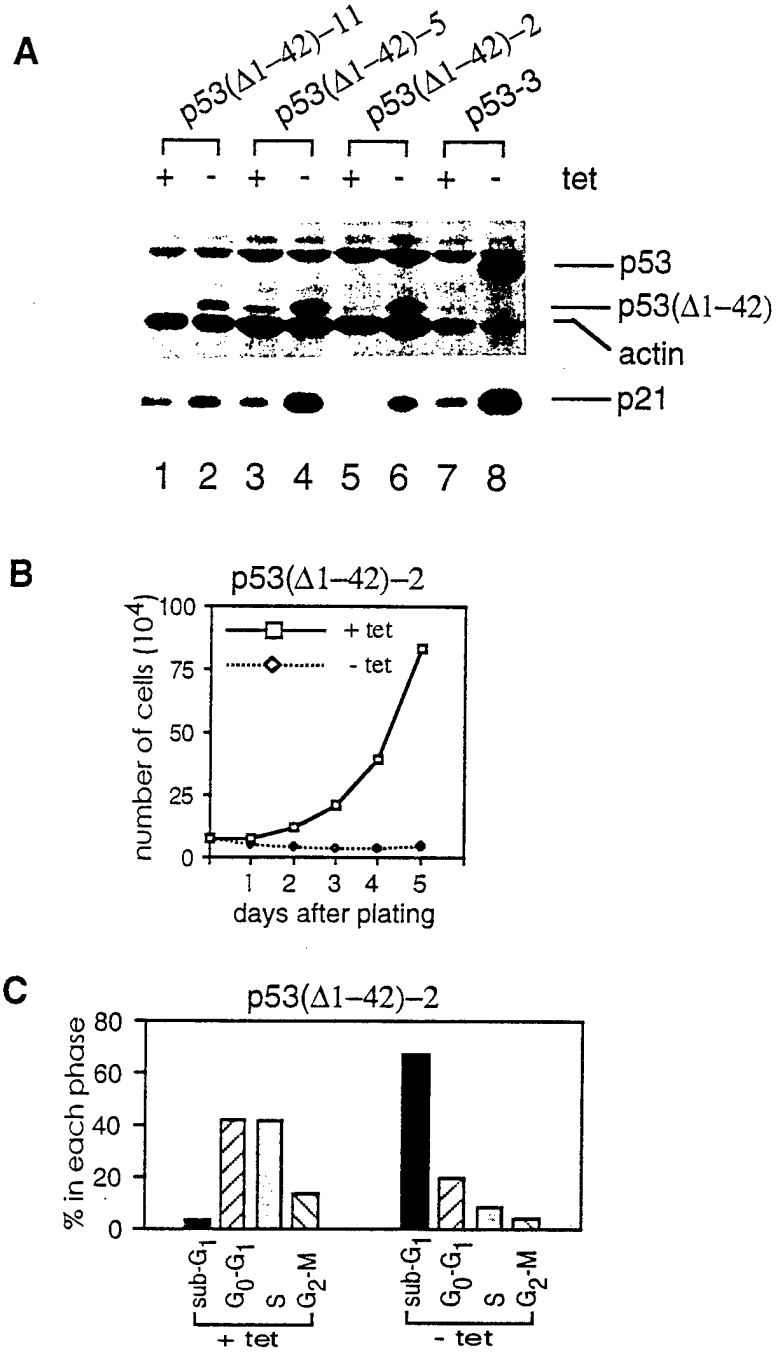
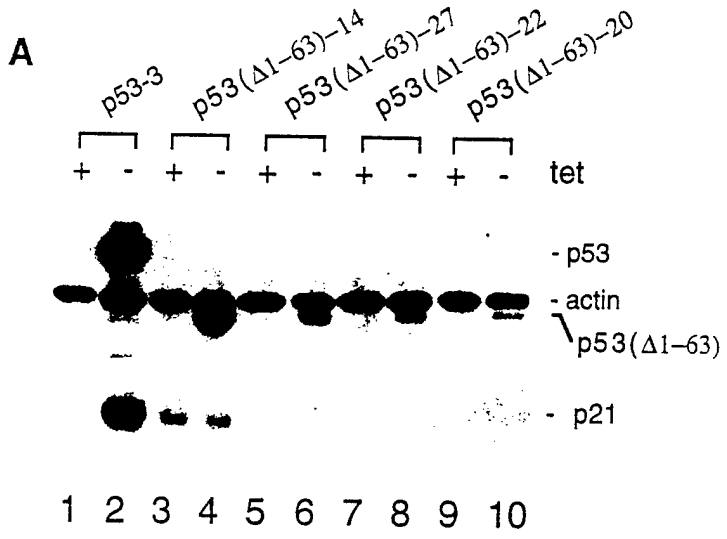
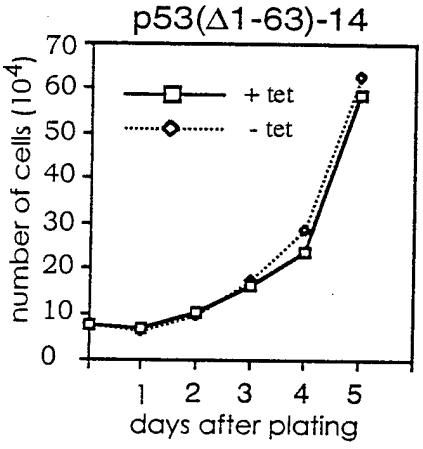

A
p53(Δ1–42)–11
p53(Δ1–42)–5
p53(Δ1–42)–2
p53-3
+ - + - + - + -
tet
p53
p53(Δ1–42)
actin
p21
1 2 3 4 5 6 7 8
B
p53(Δ1–42)–2
number of cells (10^4)
100
75
50
25
0
+ tet
- tet
1 2 3 4 5
days after plating
C
p53(Δ1–42)–2
% in each phase
80
60
40
20
0
sub-G1
G0-G1
S
G2-M
+ tet
- tet

Figure 16


A
p53-3
p53(Δ1–63)–14
p53(Δ1–63)–27
p53(Δ1–63)–22
p53(Δ1–63)–20
+ - + - + - + - + - tet
- p53
- actin
p53(Δ1–63)
- p21
1 2 3 4 5 6 7 8 9 10


B
p53(Δ1-63)-14
number of cells (10^4)
70
60
50
40
30
20
10
0
+ tet
- tet
1 2 3 4 5
days after plating

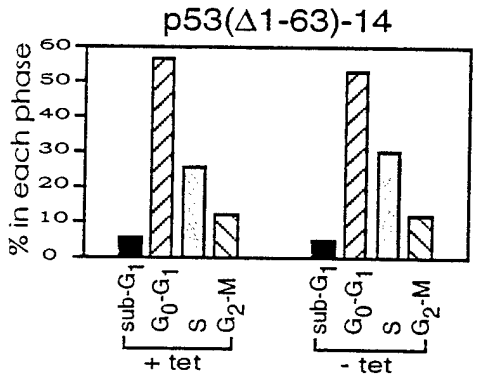

C
p53(Δ1-63)-14
% in each phase
60
50
40
30
20
10
0
sub-G1
G0-G1
S
G2-M
+ tet
- tet

Figure 17

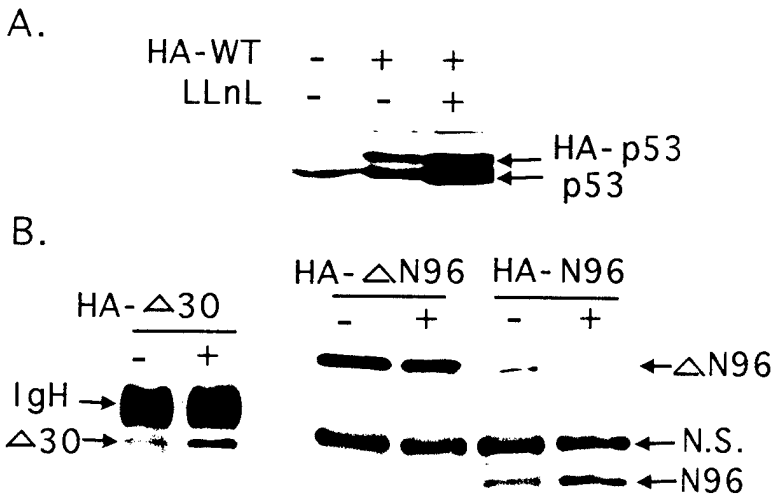


Figure 18

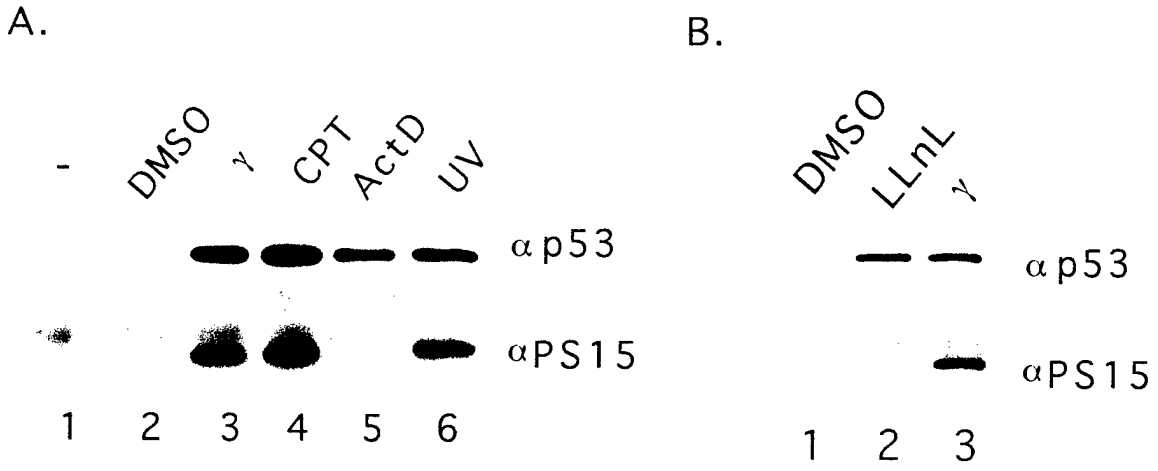

A.
-
DMSO
γ
CPT
ActD
UV
αp53
αPS15
1 2 3 4 5 6
B.
DMSO
LLnL
γ
αp53
αPS15
1 2 3

Figure 18

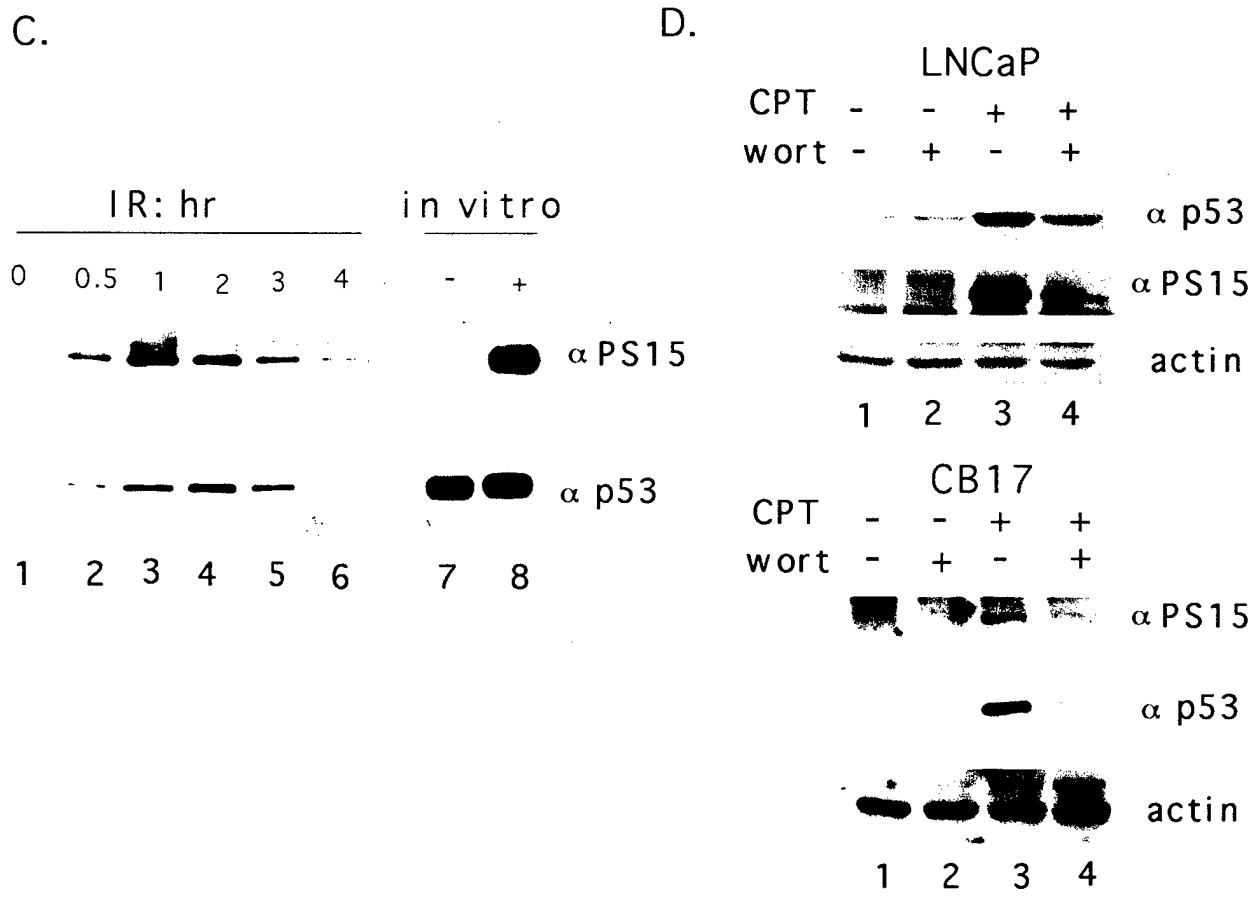

C.
IR: hr
in vitro
0 0.5 1 2 3 4
- +
αPS15
α p53
1 2 3 4 5 6 7 8
D.
LNCaP
CPT - - + +
wort - + - +
α p53
αPS15
actin
1 2 3 4
CB17
CPT - - + +
wort - + - +
αPS15
α p53
actin
1 2 3 4

Figure 19